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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: WO 91/17769
A61K 39/395	A1	(43) International Publication Date: 28 November 1991 (28.11.91)

(21) International Application Number: PCT/US91/03406

(22) International Filing Date: 21 May 1991 (21.05.91)

(30) Priority data:

527,203	22 May 1990 (22.05.90)	US
688,654	19 April 1991 (19.04.91)	US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NOVEL METHODS FOR PRODUCING ANTIGEN-SPECIFIC, HIGH-AFFINITY HUMAN MONOCLONAL ANTIBODIES

(57) Abstract

The present invention relates to advances in monoclonal antibody production, and particularly, in culturing methodology preparatory to hybridoma formation. Specifically, retention of lymphoid fragments in the culture systems results in predominantly IgG-secreting, long-lived cultures.

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NOVEL METHODS FOR PRODUCING ANTIGEN-SPECIFIC, HIGH-AFFINITY HUMAN MONOCLONAL ANTIBODIES

Reference

This is a continuing application under 35 U.S.C. 120/121 of application U.S.S.N. 07/527,203 filed 22 May 1990.

Acknowledgement

This invention was made with U.S. government support in the form of a grant from the National Institutes of Health. The government has certain rights in this invention.

Field of the Invention

This invention relates to the field of biotechnology, and more specifically, is directed to a novel method of preparing and culturing *in vitro*, human lymphocytes having particular characteristics such that antigen-specific IgG as well as IgM human monoclonal antibodies are obtainable with useful affinities suitable for clinical application.

Background of the Invention

Much research activity has focused on endeavors to produce monoclonal antibodies for evident industrial applicability in the field of medicine. Kohler and Milstein pioneered the production of specific

monoclonal antibodies by somatic cell hybridization techniques (Nature 356, 495 (1976)). That technique involved fusing lymphocytes that had been immunized in vivo against a particular antigen with a fusion partner such as myeloma cells. The fused cells were then expanded in a selection medium. The surviving hybridomas were assayed for the production of the desired monoclonal antibodies and the selected positives were subcloned, expanded and frozen.

Initial research concentrated on in vivo immunization and those efforts continue. Substantially less effort needs to be expended for the production of monoclonal antibodies if a reproducible method for in vitro induction of immune responses were to become available. Accordingly, in vitro immunizations were pursued.

Borrebaeck and coresearchers at the University of Lund in Sweden are believed to be the first to have reported the use of mixed lymphocyte cultures (MLC) by co-culturing histoxenogeneic mouse lymphocytes. In a first paper, they cultured in such a way so as to prevent cell aggregation. See Borrebaeck, Scand. J. Immunol. 18, 9 (1983).

In a later paper, they extended their analysis of the requirements for B cell growth and differentiation factors derived from such mixed lymphocyte cultures. Again, they worked with lymphocytes obtained from histoxenogeneic mice spleen tissue. Although it is not clear exactly how they prepared their spleen tissue, they do report removing non-adherent cells and recovering bound cells. They report the importance of supplementing their MLC lymphocytes with derived lymphokines and these were the preparations used in their studies. They report that

without the support of growth factors, such as lymphokines, no significant antigen-specific immune response could be recorded from the in vitro immunization.

U.S. Patents 4661586 and 4816249 are relatively recent additions to the art but are limited in example to the use of immunized mice spleen cells which were then fused to immortal lines.

These Kohler and Milstein procedures and in vitro immunization methods used lymphocytes of rodent origin. In human clinical applications, the use of such non-human monoclonal antibodies is not optimal in that rodent antibodies are foreign to the human host, and therefore could be expected to induce host immunity responses and subsequent reductions of therapeutic efficacy.

Consequently, research efforts included endeavors to produce human monoclonal antibodies. However, human cells are difficultly cultured in vitro. A 1986 review is provided by Larrick, et al., Journal of Biological Response Modifiers 5, 379 (1986). See also James et al., J. Immun. Methods 100, 5 (1987). This review article concluded by speculating on whether human monoclonal antibodies would prove to be efficacious replacements of the rodent monoclonal antibodies, depending largely on the ready availability of such human monoclonal antibodies in clinically useful purified form, the problems of maintaining a stable culture of human cells effectively immunized against a specific antigen being the major stumbling block.

Croce, et al., Nature 288, 488 (1980) and Olson and Kaplan, PNAS 77, 5429 (1980) reported on applying the

Kohler and Milstein techniques to human cells. See also European Patent Application Publication No. 44722 and U.S. Patent 4668629. In the latter documents is disclosed an alternative means of producing splenocytes in either an in vivo or in vitro immunization. However, the experimental data provided for the preparation of spleen tissue by Ficoll-Hypaque centrifugation, with apparent removal of adherent cells, and the lymphocyte mononuclear cell suspension products were fused with a specific human myeloma cell line.

Similar procedures are disclosed by Larrick, et al. in U.S. Patent 4624921. See also European Patent Application Publication No. 157574 where Ficoll preparation of peripheral blood lymphocytes was also used and the lymphocytes were transformed with Epstein Barr Virus (EBV), a means engaged in by many prior researchers to produce cells that can be grown continuously, albeit often losing their ability to secrete immunoglobulins (Ig) in a relatively short period of time. European Patent Application Publication No. 62409 describes a human lymphoblastoid cell line capable of acting as a fusion partner in the preparation of hybridomas in a rather specific manner.

U.S. Patent 4451570 similarly discloses use of human cell lines for the production of human monoclonal antibodies but emphasizes the use of non-adherent lymphoid cell culture for fusion and the preparation of the lymphocyte cells with Ficoll techniques.

Patent Application WO85/02413, corresponding to European Patent Application Publication No. 0162918 report on the preparation of human monoclonal antibodies specific to human Rh(D) antigen, using

peripheral blood lymphocytes (PBLs) separated by Ficoll-Hypaque gradient. See also European Patent Application Publication No. 174204.

European Patent Application No. 292965 reports on the production of a stable, continuous human cell line that secretes less than 40 ng/ml of endogenous IgM antibodies from a Epstein-Barr virus transformed human lymphoblastoid B cell line fusion.

Yamaura et al, J. Immunol. Methods 84, 105 (1985) report on primary immunization of spleen cells combined with monoclonal antibody production of both IgM and IgG. They state that the EBV transformation step is essential for the expansion of antigen specific clones, isotope switching and subsequent hybridoma production.

For related systems, attention may also be directed to the following: Ho, et al., J. Immunol. 135, 3831 (1985); Cavagnaro, et al., Biotechniques 1, 30 (1983); Strike, et al., J. Immunol. 132, 1798 (1984); Wasserman, J. Immunol. Methods 93, 275 (1986); Hoffmann, PNAS 77, 1139 (1980); and Pollock, et al., in vitro Immunization in Hybridoma Technology, Elsevier Science Publishers, Amsterdam, 1988, page 277. The Strike, et al paper uses an immunization of an allogeneic culture of tonsillar lymphocytes.

Borrebaeck, et al., PNAS 85, 3995 (1988) later reported on the preparation of human-human hybridomas for the production of human monoclonal antibodies from peripheral blood lymphocytes immunized in vitro against T cell dependent antigens. Again, their in vitro immunizations required support by various known growth and differentiation factors. See also the

related PBL papers Danielsson, et al., Immunology 61, 51 (1987) and Borrebaeck, J. Immuno. Methods 123, 157 (1989).

In contrast to the results herein, the Banchereau et al., Science 251, 70 (1991) lymphocytes die after about 10 weeks.

Finally, the coinventors of the present application disclosed certain preliminary findings of their research which led to the present invention. Those preliminary findings were published in a meeting held 23 to 30 April 1988 and were followed by proceedings published in early 1989 in Human Tumor Antigens and Specific Tumor Therapy, Alan R. Liss, NY, NY (1989), p. 147. The conditions described were insufficient to produce high-affinity, antigen-specific monoclonal antibodies presumably because the numbers of adherent cells, now known to be essential, were depleted.

Since publishing these findings, the present inventors expanded their research considerably and have now produced methodology enabling the preparation of human monoclonal antibodies that exhibit useful affinities for specific antigens, including human antigens, and is the subject of the present invention. An effective number of these monoclonal antibodies are IgG, an antibody class not frequently obtained from primary in vitro immunizations. The present invention is believed to represent a major step toward the production in sufficient quantities of stable efficacious human monoclonal antibodies befitting their use in a clinical setting for the assaying or treatment (of human beings) against antigens specific for a particular disease state.

Summary of the Invention

The present invention provides in vitro cultures comprising human lymphocytes that are obtained from lymphoid tissue, having essential numbers of autologous accessory, including adherent, cells. These lymphocytes are immunized in vitro against a specific antigen. In particular, the lymphocyte preparations and culturing conditions are such that the resultant monoclonal antibodies obtained after immortalization include effective numbers of IgG having useful affinities to the specific antigen, i.e., of at least about 5×10^7 liters per mole (an expression representative of an affinity constant, K, that is equal to 1/C where C is concentration or M (mole) per L (liter). The present system produces effective numbers of such monoclonal antibodies without requiring supplementation by various growth or other factors that have heretofore been reported as necessary for production of antigen-specific antibodies. A particular embodiment of the present invention involves coculturing allogeneic lymphocyte cells, that is, for example spleen cells taken from histoincompatible individuals.

The present invention is directed to such lymphocyte preparations and culturing methods useful to produce such monoclonal antibodies in all aspects, including particularly, the methods of lymphocyte preparation, the resultant cultures themselves, and essential components, and the stable continuous cell lines that produce the antigen-specific monoclonal antibodies prepared, for example, by fusion with an immortal fusion partner or by means of recombinant DNA. For example, cell lines can be operatively transfected with DNA encoding the variable region of the antibody desired. It also is directed to the human monoclonal antibodies produced from such stable continuous lines

as well as a process for preparing such antigen-specific monoclonal antibodies, such as by co-culturing allogeneic spleen lymphocytes.

Further, the present invention is directed to antibodies obtained as described herein linked to a material capable of modulating cell growth, or with a reporter molecule. In the former case, the monoclonal antibody hereof derived by immunization with, for example, a tumor antigen, is useful to site specifically target a tumor cell receptor for treatment of the cell with a material capable of killing it or arresting its growth. Similarly, where a monoclonal antibody hereof would be linked to a reporter molecule it could serve as a diagnostic tool identifying presence of cells producing antigen specific to a disease state.

It has been found that the use of three specific means of preparation of lymphocytes provide the minimum number of accessory cells that support this invention. One method is to discard the commonly used method of Ficoll treatment to isolate lymphocytes, instead using a hypotonic lysis of red blood cells, such as with ammonium chloride. The second means is to minimize conditions that are conducive to cell binding to exposed vessel surfaces by minimizing room temperature incubations, thereby reducing loss of adherent accessory cells during preparation. The third means is to retain, and/or supplement with, small fragments of lymphoid tissue in the final cell suspension prior to freezing. These expedients, specifically detailed infra. and their equivalents, are the enabling process predicates of the present invention.

The presently described invention is further specifically characterized by the following: 1. The process described will support a primary immunization response by human lymphocytes in vitro. The novel aspect is that the conditions which underlie the specific immune responses support development of antigen induced IgG- as well as IgM-secreting B cells. 2. This process, when applied to human lymphocytes in good condition, will support development of a human immune response to human as well as to foreign antigens. 3. Human lymphocytes, immunized and fused according to the protocols described herein, are capable of producing hybrid clones at a frequency (# hybrids produced/million lymphocytes fused) 5-10-fold higher than previously reported for in vitro immunized human lymphocytes. Of the hybridomas produced, a minimum of about 3 to 10 percent are antigen-reactive, depending upon the nature of the antigen. Of the antigen-reactive monoclonal antibodies produced, about 20 - 90% have been IgG.

Thus, it will be apparent that following the teachings of the present invention, one endeavors to use techniques that ensure the presence of high numbers of accessory, including adherent, cells in the human lymphocyte culturing. The end points of such successful techniques, or equivalents to those specifically taught herein, are the production of monoclonal antibodies being antigen specific and exhibiting useful affinities in the order of at least about 5×10^7 liters per mole and having effective numbers of the IgG class. These endpoints are measured by means known in the art, e.g., via competition ELISA assay.

Present teachings emphasizing the essential presence of large numbers of accessory, including adherent, cells in the culture suspension are notably followed by specifically including fragments of lymphoid tissue. One can accomplish this by using techniques which will not exclude such fragments and/or by specifically supplementing the culture with lymphoid tissue fragments. While not intending to be bound to any specific theory, experimental observations with systems containing deliberate quantities of lymphoid tissue reveal consequential multiple accessory cells. These cells appear to form part of loose aggregates of undefined material that within weeks develop into tight forms having a well-defined border. These are thought to be putative germinal centers and are defined as "splenoids." These splenoids develop out-pockets from the membrane, "blebs", that themselves may migrate to form additional accessory cell aggregates and so forth. It has further been observed that such systems support IgG secretion for months and depict class switch from IgM to predominantly IgG within that time.

Thus, use of lymphoid fragments in the culturing systems herein results in predominantly IgG-secreting culture that are long-lived upwards of six months and beyond.

Detailed Description

1. Particularly Preferred Embodiments

As a contemplated best mode disclosure, there are described successful results producing human monoclonal antibodies specific to the human and horse ferritin antigens. The specific culturing conditions that enable the production of the antigen-specific, high-affinity monoclonal antibodies hereof are similarly detailed. It will be understood, however,

that one of ordinary skill in the art having been provided the present disclosure will well enough know how to employ the present invention in the preparation of other human high-affinity monoclonal antibodies having specificity to other antigens. Similarly, alternative human lymphocytes other than splenocytes may be employed by following the teachings of the present invention which were initially applied with that particular lymphoid tissue. Moreover, other fusion partners may be employed by following the teachings hereof employing a particular fusion immortal line.

It is also foreseeable from the teachings of the present invention that having provided methodology for producing antigen-specific monoclonal antibodies, that means are available in the art to characterize and sequence the variable region. Armed with that information, one can apply known recombinant DNA techniques to clone the DNA of the variable region or of the entire Ig molecule bearing such a variant region and introduce such DNA operatively into stable recombinant host cells for expression and collection of the encoded Ig molecule. Similarly, the DNA may be amplified using, for example, polymerase chain reaction (PCR) techniques prior to or simultaneously with its expression in a suitable stable host. All of this is enabled by virtue of the present disclosure which provides the means for generating immunized B lymphocytes bearing high-affinity, antigen-specific human immunoglobulins.

The present, specific <u>in vitro</u> immunization culturing method, which (as noted above) can be expanded to equivalent means by those skilled in the art following the teachings hereof, essentially provides lymphoid tissue preparations that contain essential

numbers of accessory, including adherent, cells. This result in effect produces cultures that functionally reproduce in vivo environments capable of supporting primary immunizations and generation of antigen-specific IgG-bearing B cells and IgG-secreting plasma cells.

In the in vivo late primary or secondary response (booster), antigen exposure is immediately encountered with specific antibody forming antigen-antibody complexes referred to as iccosomes (immune complex coated body) and is trapped in the lymphoid organs on the surface of the follicle dendritic cells. Expansion and maturation of antigen-specific B cells is thought to occur within so-called germinal centers and include the phenomena of class switch (from immunoglobulin IgM to IgG, IgA and IgE), as well as affinity maturation (through point mutations in the variable region). These two phenomena are considered to be the essence of effective late primary and secondary responses.

As noted, the above-described in vivo immune response system is believed to be functionally reproduced by the present invention in vitro. Conditions reproducing, at least in part, follicle and germinal center formation are suspected to be required for the important phenomena of class switching and affinity maturation for most effective production of higher affinity, antigen-specific (preferably IgG) antibodies. The elements thought to be required for optimal responses in vitro are the presence of: B cells bearing surface immunoglobin through which they can recognize and bind to specific antigens, T helper cells that stimulate the proliferation of B cells by localized secretion of various factors, macrophages that ingest, process and present antigens,

appropriate lymphokines that are produced by macrophage and T helper cells, and the all important presence of dendritic cells that promote homologous and heterologous mixed lymphocyte response (MAR) reactions and cellular aggregation.

In the preferred embodiments of the present invention, spleen cells are employed. These spleen cell cultures are prepared in a mild fashion using, for example, ammonium chloride lysis instead of the common prior art use of ficolling that serves to separate red cells from white cells. The preparation and culturing of lymphocytes will minimize conditions that are conducive to cell binding to vessel surfaces. In an embodiment, mixed instead of single spleens are employed in allogeneic cultures, providing initially highly responsive systems. These combined processes, that are described in respect of the preferred embodiments hereof, provide all of the elements thought to be essential for the successful in vitro primary followed by secondary responses at least in part representative of those that are produced in vivo in a human organism upon encountering antigen. As noted above, aspects of these essential elements are provided in the preferred embodiments by the use essentially of human lymphocytes obtained from lymphoid tissue via methodology that preserves essential numbers of autologous accessory, including dendritic and adherent cells.

This is made possible primarily because of specific elements that may be employed in the method of lymphoid tissue, e.g. splenocyte, preparation:

a. gentle dissociation of lymphocytes and accessory cells from cut pieces of lymphoid tissue by manual manipulation, e.g., with forceps and the

(rubber-tipped) blunt end of a plunger from an appropriate sterile plastic syringe.

b. removal of red blood cells by gentle hypotonic lysis instead of by ficolling.

c. prevention of loss of adherent cells by minimizing exposure to flat surfaces at temperatures above about 4° C.

d. retention of, and/or supplementation with, small (less than or about 1 cubic millimeter) fragments of splenic connective tissue within the final cell preparation.

Collectively, actions a-d give rise to a mixture of healthy splenocytes and accessory cells, including macrophages, follicular and lymphoid dendritic cells, and fibroblasts. This combination of cells, in the state of activity provided by their gentle dissociation from whole spleen, more closely reproduces the in vivo splenic environment than any combination of lymphocyte preparation steps or culture methods previously described.

The systems hereof manifest splenoids that are thought to be in vitro equivalents to in vivo germinal centers of the late primary or secondary stages of immune response, and manifest the results of such response by class switch and long-lived IgG secreting cultures.

A major use of the described process is the production of immortal, continuously secreting hybridoma clones which produce human monoclonal antibodies specifically reactive with almost any antigen of interest, including human antigens.

Thus, the present invention provides for the first time an in vitro immunization culturing system that

produces relatively high numbers of high-affinity, antigen-specific monoclonal antibodies when converted into stable continuous cell lines, that are useful in a clinical setting.

2. Description of Parameters.

The present invention is predicated on the finding that the method of preparing the lymphocytes for culture and immunization in vitro is essential. The essential aspects employ methodology ensuring the retention of essential numbers of accessory cells within the lymphocyte cultures. Such accessory cells are necessary for reproducing at least in part the lymphoid follicle type of environment that supports efficient antigen presentation, class switching and affinity maturation. The presence of essential numbers of autologous accessory cells is manifested by the presence of an "essential number" of adherent cells in the culture. It has been found from research results that optionally the numbers of non-lymphocyte (non-T, non-B) cells, i.e., that population of cells containing essential accessory cells, in the final preparation should not be less than about 10% of the total cell number. It has also been observed that the numbers of essential adherent monocyte/macrophage cells in the lymphoid tissue chosen for culturing should not be less than about 2% of the total cell population. These numbers were arrived at by labelling monocytes and macrophages with a specific monoclonal antibody, tagging with a fluorescent label and determining the percent of labelled cells by fluorescence activated cell sorting (FACS) analysis.

It will be understood that culturing techniques can be varied from those specifically disclosed herein so as to ensure the retention of effective numbers of

accessory cells thought to be essential. Further, it may be found with further research not requiring undue experimentation that the numbers of adherent cells disclosed as essential herein may vary somewhat outside of the minimum range given; hence, the use of the term "about" when defining that limit.

It has been found via the research herein that the culturing conditions that use essential numbers of adherent cells provide effective numbers of IgG class immunoglobulins. It is believed, based upon the present research, that a minimum of about 10 percent of the monoclonal antibodies hereof being of the IgG class would be an "effective number"; a 50 to 80 percent level would be considered most effective for identification of clinically useful monoclonal antibodies. Again, it will be understood that varying the culturing conditions within the general scope of the present invention may provide numbers of IgG molecules that are effective but somewhat outside of the range as specified. It is considered that where those numbers are effective, there are generated sufficient antigen-specific IgG monoclonal antibodies such that antibodies having useful affinities and good specificity can be selected. Hence, the use of the term "about" when expressing those values.

3. Definitions and General Procedures

In a specific process hereof, splenocytes, obtained by careful dissection from human spleen tissue, are immunized, i.e., specifically stimulated, by microgram quantities of either xenogeneic or allogeneic antigen. The stimulated B cells are then collected at a specific time after exposure to antigen and fused at high efficiency to a heteromyeloma human fusion partner, K6H6/B5 (publicly

available; see also J. Immunol. Meth. 89:61-72, 1986), to produce hybrid clones which secrete human monoclonal antibodies specifically reactive with the immunizing antigen. Specifically immunized cultures may also be depleted of antigen and allowed to secrete antibody which may then be detected by conventional ELISA techniques. Post-immunization assay allows semi-quantitative evaluation of the responses and of the classes of immunoglobulin produced under a wide variety of immunization conditions. These results are then used to identify optimal conditions for immunization prior to fusion and to estimate the results of subsequent fusions.

By the term "in vitro immunization culture" is meant a culture in which the immunization with specific antigen is not done in man or any other animal; instead, a culture derived from tissue of human origin is cultured ex vivo.

By the term "lymphoid tissue" from which the human lymphocytes hereof are obtained for culturing is meant any human lymphoid tissue that can be expected to have large numbers of T-/B-cells, and accessory cells, including spleen tissue, tonsils and lymph nodes.

By the term "essential numbers" of autologous adherent cells herein is meant numbers that prove to produce IgG monoclonal antibodies in accord herewith that have useful affinities specifically to a given antigen, both non-human and notably human. These results are preserved herein by the expedient of using lymphoid tissue fragments in the culture system. Hence, the term has a functional definition within an approximated range defined above and can be attained by specific, preferred means.

By the term "autologous accessory cells" herein is meant non-T, non-B cells which support or enhance immunization responses, derived from the same individual from which the T and B lymphocytes are obtained.

By the term "specific antigen" is meant any antigen that is desired for specific-reaction with a human monoclonal antibody hereof including various tumor antigens such as ferritin, carcinoembryonic antigen (CEA), TAG 72, etc.

By reciting herein that the culturing of the present invention does not require "supplementation with growth or other factors", such as lymphokines, that have heretofore been considered essential is meant that the conditions described herein make it unnecessary to supplement exogenously the cultures with these materials. That is not to imply that the invention proscribes the supplementation with such materials, only that it is unnecessary by virtue of the advance of the present invention.

In the term "at least about" in respect of the affinity of the monoclonal antibodies hereof, by the component "at least" is meant that the value specified represents a currently believed threshold value above which the monoclonal antibodies would find utility in a clinical setting. The component "about" in respect of this term is intended to convey some latitude in the interpretation range of the term in question, given the inherent variances of biological systems such as differences in host cells employed, fusion partners employed, antigens chosen and in culturing conditions from those specifically disclosed herein. It will be understood that it is considered within the skill of the art to vary the

procedures and produce clinically useful human monoclonal antibodies in accord herewith that may differ in value from that given for the optimum threshold affinity provided herein.

The term "effective numbers" in respect of the IgG class antibodies is defined as discussed above.

The term "multiple macroscopic aggregate forms" is used to define the observation that clumps of cells derived from lymphoid tissue, which can include B and T lymphocytes and accessory cells, begin forming within hours of incubation of culture, and are visible without magnification.

"Splenoids" develop within one to two or more weeks of culturing and have been observed where fragments of lymphoid tissue are specifically included in the cultures. They may be mimics of the in vivo germinal center of late primary or secondary immune response.

By the term "allogeneic coculture" is meant a culturing of histoincompatible tissues, e.g., spleen or other lymphoid tissue from more than one non-identical individual.

The reference to "IgG", etc. is the standard reference to immunoglobulins of one of the five known major classes of constant regions that determine the class of the immunoglobulin molecule and are referred to as IgG, IgM, IgA, IgD and IgE.

By the term "immortal fusion cell partner" is meant a cell line consisting of cells which reproduce themselves indefinitely and which, upon fusion with a lymphocyte-bearing functional immunoglobulin gene, provides a vehicle for indefinite and constant

secretion of immunoglobulin molecules coded by said gene. Such fusion partners include myeloma and plasmacytoma cell lines as well as heteromyelomas and heterohybridomas.

By the term "via recombinant means" and "harboring operatively" is meant the generally known and published methodology by which suitable host cells are transfected with DNA, preferably within vectors where the encoded DNA is linked to DNA elements that induce expression to form encoded polypeptide. Such expression vectors are generally replicable and may remain as episomes or as an integral part of the host chromosome.

See also, generally, Manitias, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1982, and various references cited therein, and in particular, Colowick, et al., Methods in Enzymology, Vol. 152, Academic Press (1987).

By the term "material capable of modulating cell growth" is meant a material that satisfies two criteria, namely, that it be specifically linkable to the antibody such as via a chelate or a direct covalent link, and that it have a cytocidal or cytostatic effect cellularly in vivo. Examples of such materials would include radioactive substances such as yttrium$_{90}$, iodine $_{131}$ and toxins such as ricin.

By the term "reporter moiety" is meant a material that satisfies two criteria, namely, that it be specifically linkable to the antibody, such as via a chelate or a covalent link, and that it provide some identification of its existence such as in a bioassay, or more commonly, in a chromaphore assay.

Examples of such moieties would include a radioactive label such as $indium_{111}$, or enzymes such as alkaline phosphatase.

Reporter moieties may be attached to the antibodies hereof according to conventional techniques known per se in the art. For example, nucleophilic groups on the antibodies such as primary amine groups may react with a fluorescent or enzymatic reporter group to form a covalent bond, or bifunctional coupling reagents known per se in the art may be employed.

Other useful reporter moieties are biotin, fluorophores, chemiluminescent moieties, enzymes or colloidal compounds. Examples of fluorophore groups are fluorescein-5-isothiocyanate, diacyl fluorescein-5 and/or 6 carboxylic acid pentafluorophenyl ester, tetramethylrhodamine-5 (and 6) isothiocyanate, eosin-isothiocyanate, erythrosin- 5-isothiocyanate, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole, succinimidyl 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminododecanoate, 7-hydroxycoumarin-4-acetic acid, 4-acetamido-4′-isothio-cyanatostilbene-2-2′-disulfonic acid, 9-chloroacridine, p-nitrophenyl 1-pyrenebutyrate, 9-anthracenepropionic acid, or 2-anthracenesulfonyl chloride.

Enzymic reporter moieties include β-galactosidase, horse radish peroxidase, alkaline phosphatase, dehydrogenases, luciferase and carbonic anhydrase.

4. Figure Legends

Figure 1. Ferritin-reactive IgM antibody secretion by in vitro primed human lymphocytes: comparison of single (A,B) and allogeneic, mixed (A+B) cultures.

Human splenocytes were prepared without depletion of adherent cells as described infra and cultured at a density of 3 x 10(6) cells/ml in the presence of absence of 1 ug/ml horse spleen ferritin. Allogeneic cultures consisted of a 1:1 mixture of cells prepared from spleens A and B.

Figure 2. Ferritin-reactive IgM antibody secretion by in vitro primed human lymphocytes: influence of inactivating X-radiation on specific immune responses by single and allogeneic mixed cultures.

Human splenocytes were prepared without depletion of adherent cells as described infra and exposed to 2000 rays of x-radiation within 1-2 hours of preparation. Within one hour of irradiation, syngeneic or 1:1 co-cultures of normal and/or irradiated cells were primed with 0 or 2 ug/ml horse spleen ferritin for 3 days. After removal of ferritin by washing, the cells were cultured for an additional 2 days. The supernatants were collected and analyzed by ELISA for reactivity with adsorbed ferritin.

Figure 3. Ferritin-reactive IgM antibody secretion by in vitro primed human lymphocytes: time dependence of ferritin priming and secretion of ferritin-reactive antibody.

Human splenocytes were prepared without depletion of adherent cells and cultured at 1.5 x 10(6) cells/ml with the concentrations of horse spleen ferritin indicated in the figure (0 - 1.0 ug/ml). The top row of figures represents cells which had been primed for 1 day, washed free of antigen, and cultured for an additional 1, 2, 3, or 5 days, as indicated, to allow antibody secretion in the absence of ferritin. The second, third and fourth horizontal rows of figures

represent antigen priming for 2, 3, and 4 days, respectively, after which antigen was removed and the cells were cultured without ferritin for an additional 2, 3, or 5 days, as indicated. Culture supernatants were collected and analyzed by ELISA for reactivity with adsorbed ferritin.

Figure 4. Ferritin-reactive IgM and IgG antibody secretion by in vitro primed human lymphocytes: time dependence of secretion after a 3 day immunization.

Human splenocytes were prepared without depletion of adherent cells. Duplicate wells were cultured at 3 x 10^6 cells/ml with or without 1 ug/ml horse spleen ferritin for 3 days. Ferritin was removed by washing and the cells were cultured for an additional 13 days without ferritin. Supernatants were collected for ferritin-reactivity analysis by ELISA at days 5, 7, 9, 11, 13, and 16. After each collection the cultures were refed with 1.5 ml culture medium. Culture supernatants collected on a given day contained primarily immunoglobulins secreted between the previous refeeding and the time of supernatant collection. Open and shaded areas represent IgM and IgG responses, respectively.

Figure 5. Affinity/avidity analysis of ferritin-reactive IgM antibodies secreted by in vitro primed human lymphocytes. Human splenocytes were prepared without depletion of adherent cells. 1:1 co-cultures were primed with 0 or 1 ug/ml horse spleen ferritin for 3 days followed by washing to remove ferritin and 2 additional days of culture in the absence of ferritin. Ferritin reactivity of secreted antibody was analyzed by ELISA and relative affinity/avidity was estimated. Standard error bars not shown are contained within the symbols.

Figure 6. Ferritin-reactive IgM antibody secretion by human lymphocytes primed in vitro in the presence or absence of adherent cells: comparison of responses to horse and to human ferritin.

Human splenocytes were prepared without depletion (B and D) or with depletion (A and C) of adherent cells. 1:1 co-cultures of cells from 2 spleens were primed with the indicated concentrations of horse spleen ferritin (A and B) or with human (C and D) ferritin for 3 days. After removal of ferritin by washing the cells were cultured for 2 additional days and the supernatants were collected and analyzed by ELISA for reactivity with adsorbed ferritin.

Figure 7. Ferritin-reactive antibody secretion by in vitro primed human lymphocytes: comparison of IgM and IgG responses to horse and human ferritin in the presence and absence of adherent cells. Human splenocytes were prepared with or without depletion of adherent cells. 1:1 co-cultures were primed at 3 x 10^6 cells/ml with the indicated concentrations of horse or human ferritin for 3 days. After removal of ferritin, the cells were cultured for 2 days and supernatants were analyzed by ELISA for ferritin reactivity.

Figure 8. Antigen-reactive IgM antibody secreted by human lymphocytes primed in vitro with purified murine monoclonal antibodies, No. 1 or No. 2.

Human splenocytes were prepared without depletion of adherent cells and cultured at 3 x 10(6) cells/ml for 3 days with the concentrations of purified murine monoclonal antibody indicated in the figure. After removal of antigen by washing, the cells were cultured for an additional 2 days. The supernatants

were analyzed for reactivity with the immunizing antigen by ELISA. Antibodies 1 and 2 differed in their light chain components (#1 contained a lambda light chain and #2 contained a kappa light chain).

Figure 9. Comparison of reactivity by ELISA of a ferritin-reactive, specific monoclonal antibody (A), with a ferritin-reactive, non-specific monoclonal antibody (B).

Culture supernatants were harvested from terminal cultures of each clone grown in 24 well plates and assayed by ELISA as described infra. Immunoglobulin concentrations of culture supernatants were in the range of 1-10μg/ml. Reactivities with increasing concentrations of ferritin coated to the assay plate (values shown along the X-axis) were compared with reactivities to increasing concentrations of ferritin-unrelated proteins as shown in the figure.

Figure 10. Titration of the ferritin reactivity of two purified anti-ferritin human monoclonal antibodies. Monoclonal antibodies were purified by affinity chromatography on protein G and ELISA analysis of the indicated concentrations of antibody were carried out as described infra.

Figure 11. Affinity analysis of selected ferritin-specific IgG human monoclonal antibodies by competition ELISA.

This analysis was performed as described infra. The first graph represents the degree of inhibition of antibody binding to the ELISA plate (Y-axis) as a function of the concentration of competitive ferritin in solution (X-axis). Ao represents antibody reactivity in the absence of soluble antigen (in the

form of absorbance at 490 nm produced by ELISA analysis of the incubation mixture) and A represents reactivity of the antibody incubated in the presence of the indicated concentrations of soluble antigen. The second graph represents a Klotz plot analysis of the data presented in the first panel where 1/v corresponds to Ao - A/Ao and ao represents the total concentration of free antigen. The open circles represent monoclonal antibody 14.2.2.59, produced from lymphocytes immunized with horse ferritin, having a calculated Kd = 0.86×10^{-8} M. The filled squares show monoclonal antibody 21.1B.9, produced from lymphocytes immunized with human ferritin, having a calculated Kd = 1.90×10^{-8} M.

Figure 12 represents a time course of cumulative Ig secretion from a long-term splenocyte culture in a 24-well plate prepared in accord with the present invention preserving multiple numbers of accessory cells, for example by insuring presence of lymphoid fragments during culturing. The mature immune responses of class switch (from total IgM to IgG) and longevity are evident.

Figure 13 represents the antigen reactivity (reactivity with ferritin) of the immunoglobulin (Ig) secreted by a long-term splenocyte culture in a T25 flask, showing specific antigen reactivity of the Ig secreted. The data in Figures 12, 14 and 15 represent cumulative, total Ig secretion. The data in Figure 13 represents only that component of the secreted Ig which reacts with the immunizing antigen, ferritin, and it is not cumulative. The OD values shown represent the difference in ferritin reactivity between primed and non-primed control cultures. These data show that ferritin stimulated the secretion of ferritin-reactive IgM at early time

points, 0-25 days, and that ferritin significantly stimulated ferritin-reactive IgG only after the second boost, at 70-85 days. The fact that antigen-reactive IgM secretion was stimulated by ferritin early in the life of the culture, whereas antigen-reactive IgG secretion was stimulated by ferritin only after boosting constitutes additional evidence of in vitro class switching.

Figure 14 represents a time course of cumulative, general IgM secretion from a long-term splenocyte culture in a T25 flask. The influence of a priming antigen, horse spleen ferritin, on the rate of secretion is shown. The ferritin-primed cultures showed an increased rate of IgM secretion over the first 0-30 days relative to the control culture. After 30 days the rates of both cultures appeared fairly similar until shortly after the second boost when the rate of the primed/boosted culture again appeared to increase relative to the control culture. Shortly after 100 days, the IgM secretion rate of the control culture showed a sharp increase. It is thought that this increase in the control culture resulted from a general contamination with mold which became evident at approximately 110 days and the culture had to be discarded. (The rate of secretion is calculated from the slope of the line, which is defined as the vertical rise divided by the horizontal distance spanning the rise. The steeper the slope, the greater the rate.)

Figure 15 represents a time course of cumulative, general IgG secretion from the same long-term splenocyte culture shown in Figure 13. The influence of the priming antigen, horse spleen ferritin, on the rate of IgG secretion is shown. Ferritin had no significant effect on the rate of IgG secretion 0-40

days, in contrast to the time when ferritin had the greatest effect on general IgM secretion. At approximately 44 days the rate of IgG secretion in the primed culture showed a small but significant increase relative to the control culture. At 72 days, just after the second boost followed by a wash, the rate of IgG secretion in the primed culture increased dramatically relative to the control culture. The fact that ferritin primarily stimulated IgM secretion at early times and primarily stimulated IgG secretion at later times is evidence of <u>in vitro</u> class switching.

Figure (photomicrograph) 16 shows underlying adherent cells, the outgrowth of which supports development of secondary structures. Also shown are three different secondary structures: (1) a medium sized one on the right having a large, dense center, (2) a medium sized one on the upper left having a small dense, dark center, from which looser material radiates outwardly, and (3) a small one on the lower left having a small dense, dark center and a small amount of looser material radiating outwardly.

Figure (photomicrograph) 17 shows two examples of secondary structures: (1) on the right is a denser, darker, bounded structure with light blebs appearing on the top, right; and (2) a less dense, lighter, non-bounded structure, apparently consisting of a loose collection of larger, brown cells is shown on the left, with individual brown cells on the bottom and to the left, either spreading out or in the process of aggregating.

Figure (photomicrograph) 18 shows a secondary structure on the right, which has not yet developed a dense, dark center and which rests on an underlying

layer of adherent cells (not visible under the layer of lymphocytes) and surrounded by many small, either round or irregularly shaped cells which appear to be lymphocytes. Dense collections of these small cells are occasionally observed surrounding or adjacent to the depicted secondary structures suggesting that the secondary structure may be a site of multiplication of these small lymphoid cells.

The variety of cells and structures shown in Figures 16 to 18 develop with time as the cultures mature. Outgrowth of adherent cells and development of secondary structures begins at 1-2 weeks after splenic fragments are seeded into flasks or plates and continues for a minimum of several months. Antibody secretion is not observed without outgrowth of adherent cells and development of the type of secondary structures shown. It is believed that these secondary structures are aggregates of different types of cells which represent or perform functions of <u>in vitro</u> germinal centers.

5. <u>Examples</u>

The following include protocols for the preparation, culturing, and primary immunization of human splenocytes with protein antigens. Horse spleen ferritin was used for development of these methods because this protein has been well characterized, human immune responses to horse ferritin can be compared with human responses to human ferritin, and human ferritin, in the onco-fetal form, has been characterized as a tumor-associated antigen. Thus, ferritin-reactive human monoclonal antibodies could have therapeutic application. It has also been found that conditions developed for ferritin can be applied to immunizations with other protein antigens. It is demonstrated that lymphocytes immunized as described

can be fused at high efficiency with a human/mouse heteromyeloma fusion partner to produce antigen-specific IgG as well as IgM human monoclonal antibodies.

Human splenocytes immunized in vitro with protein antigens were immortalized by high efficiency fusion with one of the heterohybrids constructed by Carroll et al., J. Immuno. Methods 89, 61 (1986), K6H6/B5, to produce specific antigen-reactive human monoclonal antibodies. Results evidenced good fusion efficiency and growth of the hybrid progeny, level of immunoglobulin secretion in the range of 0.5-50 ug/ml, stability of immunoglobulin secretion in approximately 50% of the hybrids, and production of IgG as well as IgM class antibodies.

A. Materials and Methods

Preferred Embodiments

1. Splenocyte Preparation: All steps are performed under sterile conditions:

a. Spleen tissue, obtained from accident victims within hours after surgery, was provided by the University of California San Diego (UCSD) Tissue Bank.

b. The tissue is cut into approximate 1 inch square fragments.

c. A single cell suspension in RPMI growth medium is generated by forcing spleen fragments through a 50 mesh wire screen.

d. As the cell suspension is generated, it is collected into a sterile bottle on ice and then it can be filtered through several layers of sterile

cheesecloth into a sterile glass beaker to remove larger tissue fragments.

e. The cell suspension is then transferred into 250 ml centrifuge bottles and the cells are collected by centrifugation at 1000 rpm for 10 min; the supernatant discarded and the loose pellet resuspended with a minimal volume of RPMI and transferred to a 100 ml sterile bottle.

f. The cells are exposed to hypotonic ammonium chloride for 30-90 seconds to lyse red blood cells (RBCs) and washed several times.

g. Preferably, fragments of lymphoid tissue of about 0.1 to 1.0 millimeter in size are deliberately retained in the cell suspension.

h. After the final wash, the cells are counted, resuspended into freeze medium, aliquoted into cryotubes, and frozen at about 100-600 million cells per milliliter per vial.

2. Immunization: All steps are performed under sterile conditions:

a. 100-300 million cells each from 2 separate spleen preparations are thawed by gentle shaking in a 37°C water bath; these are washed twice with RPMI.

b. The cells are resuspended in 5 ml of medium and counted.

c. The cell concentration is immediately adjusted to between 0.5-5.0 X 10(6) cells/ml and the cell suspension is then transferred to a 24 well costar plate at 2 ml/well.

d. Antigen is added at the desired concentration (0.1-10.0 ug/ml has been found as an effective range).

e. The composition of RPMI growth medium (commercially available) included the following: 10% fetal calf serum (FCS), 2 mM glutamine, and Gibco amino acids and pyruvate (1:100 dilution).

f. Exposure to antigen is allowed to proceed for at least 2 days.

g. Control cultures are also established, as described above, but are not exposed to antigen.

3. Elisa Protocols: Analysis of the immunization response:

a. After 3-5 days, the antigen is removed from the cultures as follows: the cells from each culture are resuspended by aspiration with 10 ml of RPMI + 2% FCS and transferred to a 15 ml centrifuge tube.

b. The cells are collected by centrifugation at about 1000 rpm for 10 min, and may be washed and the cells are then resuspended by aspiration with a 10 ml pipet.

c. The cells are resuspended into 2 ml of new growth medium and returned to the original culture wells to allow secretion of antibody in the absence of antigen.

d. After 2-4 days, 1.5 ml of supernatant is collected by aspiration with a pipet and stored at 4°C with 0.01% azide until assayed for immunogen-specific reactivity by conventional ELISA techniques.

e. The level of response measured for nonimmunized cultures (this represents background reactivity and is highly dependent upon culture conditions) is subtracted from the response measured using immunized cells; this difference represents a semi-quantitative measure of the antigen-driven reaction and may be used as a rough predictor of the number and predominant immunoglobulin class of the hybrid clones obtainable from fusions of lymphocytes immunized under similar conditions.

4. Immortalization: Production of hybrid clones from in vitro immunized lymphocytes.

a. After a minimum of 2 days exposure to the immunizing antigen, the cells are resuspended into 5 ml of RPMI, counted, and combined with K6F6/B5 heteromyeloma cells.

b. These cells are diluted to 50 ml with RPMI containing glutamine, pyruvate, and non-essential amino acids as described above and centrifuged at 1000 rpm for 10 min.

c. The cells are washed 1 more time with the above solution and fused and hybrids selected according to conventional fusion protocols.

Specific Protocol

Materials.

Horse spleen ferritin (F-4503), BSA (A-7906), Tween 20 (P-1379) and potassium thiocyanate (P-3011) were purchased from Sigma. Human ferritin, purified from liver carcinoma tissue, was generously provided by Dr. Jerry Klein of Johns Hopkins University, Baltimore, MD. Murine monoclonal antibodies, were generously provided by Dr. Malaya Bhattacharya-

Chatterjee (4DC6) and Dr. Ben K. Seon (SN2) of Rossell Park Memorial Institute, Buffalo, NY. Goat anti-human IgM (4102), IgG (4100), IgM-HRP (2392), and IgG-HRP (2390) were purchased from TAGO, Burlingame, CA. Primary antibodies were either purchased as purified monoclonal antibodies from Coulter Immunology, Hialea, Florida, or used as culture supernatants produced from the cell lines CRL 8001 (anti-CD3) and CRL 8014 (anti-CD8), purchased from the ATCC.

Splenocyte preparation.

Spleen tissue, obtained from accident victims within hours after surgery, was provided by the UCSD Cancer Center Tissue Bank. A single cell suspension was prepared by forcing fragments through a wire screen. The cells were collected by centrifugation at 1000 rpm for 10 minutes and RBCs were removed by ammonium chloride lysis. The remaining cells were washed, resuspended in a freezing medium consisting of 40% RPMI, 50% FCS, and 10% DMSO at a concentration of 100-300 million cells/ml, frozen in 1.5 ml aliquots, and stored in liquid nitrogen. For establishment of long-term cultures, it is essential that fragments of spleen tissue be retained in the cell suspension prior to freezing.

In vitro immunization.

All steps were performed under sterile conditions. Frozen cells from each spleen preparation were thawed by gentle shaking at 37°C and washed 2 times with 15 ml RPMI.

Two ml. immunization cultures were set up immediately after thawing and washing. For mixed cultures, each spleen contributed an equal number of cells to the final concentration. The cell suspension was then

transferred to the inner wells of a 24 well tissue culture dish at 2 ml/well. Ferritin or other antigens were immediately added at 0-10 ug/ml, as indicated. At least 4 wells were set up for each condition tested. If multiple antigen concentrations were used, one well received no antigen. This was the non-immunized, control sample, analogous to the pre-immune sera of in vivo immunizations. The remaining 3 wells each received a different amount of antigen. If only 1 concentration of antigen was to be used, 2 wells received no antigen and the remaining 2 wells received the indicated concentration.

Unless otherwise indicated, the cells were cultured with antigen for 3 days. After priming, cells from each well were gently transferred to a 15 ml centrifuge tube, washed 1 time with 15 ml RPMI + 2%FCS to remove the antigen, and then returned to the well from which they had been removed for an additional 2 days, unless otherwise indicated. Culture supernatants were collected at 5 days and the cells either discarded or refed for a second analysis of antibody production at day 7. Standard growth medium consisted of RPMI supplemented with 10 % FCS, 1% non-essential amino acids, (Irvine Scientific) 2 mM glutamine, 1 mM sodium pyruvate, 15 mM HEPES, and gentamicin.

Anti-Ferritin Assay

Anti-ferritin reactivity of antibody in the culture supernatants was estimated as follows. Purified ferritin was diluted to 10 ug/ml in .05 M sodium carbonate buffer, pH 9.3. 0.05 ml/well were incubated overnight at 4 degrees C. using 96 well round bottom Immulon I plates (Dynatech). Each supernatant was assayed in triplicate on ferritin and in duplicate on

bovine serum albumin (BSA). OD-490 values observed with BSA were considered non-specific protein reactivity and were subtracted from the OD-490 values observed with ferritin. The difference in OD values between ferritin and BSA binding is referred to as ferritin reactivity. Difference in ferritin reactivity between non-immunized and primed samples is referred to as ferritin-induced, ferritin reactivity. After coating overnight, the plates were washed 3 times with phosphate buffered saline (PBS) and blocked with 1% BSA in PBS. An alternative method giving similar results involved omission of the BSA blocking step and dilution of samples and reagents in 0.05% Tween 20/PBS. After 2 hours at 37 degrees C, the blocking solution was removed and 0.05 ml of supernatant were added to the appropriate wells. After 2 hours at 37 degrees C, the plate was washed 5 times with PBS - 0.1% Tween 20 and 0.05 ml of a 1:1000 dilution of a 1 mg/ml solution of peroxidase conjugated goat anti-human IgM were added. This secondary reagent was diluted into 10% FCS in PBS. After 45 minutes at 37 degrees C, the plates were washed 5 times with PBS-Tween and 0.150 ml/well of 0.4 mg/ml o-phenylenediamine (Sigma P-1526), dissolved in 0.05M sodium citrate buffer, pH 5.0, and 0.0175% hydrogen peroxide was added. Color development was terminated after 15-60 minutes by addition of 0.025 ml of 2.5 M sulfuric acid. OD-490 values were recorded using a Molecular Devices (Mountain View, CA) V max kinetic plate reader. Human IgG anti-ferritin reactivity was assayed similarly except that the assay wells were coated with 50 ug/ml ferritin or BSA, the protein was adhered overnight at 37°, and the secondary reagent was peroxidase-conjugated goat anti-human IgG.

Anti-murine immunoglobulin assay.

Polyclonal human anti-mouse immunoglobulin reactivity was assayed by ELISA using the basic procedure described above for detection of anti-ferritin reactivity, except that 5 ug/ml murine monoclonal immunoglobulin was used as the capture antigen.

Quantitation of human IgG and IgM.

Human IgG and IgM were quantitated by ELISA as follows. 0.05 ml/well of 1:100 dilutions of 1 mg/ml solutions of goat anti-human IgM or IgG (Tago) were adsorbed at 4°C overnight to serve as capture for the human IgG or IgM. Supernatants containing unknown quantities of immunoglobulin were serially diluted until reactivity was undetectable. Assay protocols were similar to those described for anti-ferritin reactivity, except that peroxidase-conjugated secondary reagents, goat anti-human HRP-conjugated IgM or IgM, were used at a dilution of 1:5000 instead of 1:1000. Concentrations of unknown samples were calculated from dilutions where OD values fell within the linear range of the standard curve, which was defined by standard samples of purified polyclonal human IgM or IgG, used in a range of 0.01 to 2.0 ug/ml.

Analysis of relative antigen binding affinity/ avidity values of polyclonal antibodies produced in vitro.

An assay utilizing disruption of antigen-antibody binding by thiocyanate was adapted from MacDonald et al., J Immuno. Methods 106, 191 (1988), integrated with the anti-ferritin ELISA described above, and used to characterize the quality of the in vitro immune response. After immunization, culture supernatants were incubated with ferritin bound to the assay plate and the plates washed 5 times with PBS-Tween, 0.1 ml of the indicated concentrations of

potassium thiocyanate, dissolved in PBS, were added to each well and the plate was incubated at room temperature for 15 minutes. The plates were washed 5 times with PBS-Tween and the anti-ferritin assay concluded as described above. Supernatants having high binding activities were diluted prior to assay so that ferritin binding activity would be relatively uniform from sample to sample. Each supernatant was assayed in duplicate at each thiocyanate concentration and supernatants from two separate but identical immunizations were analyzed for each condition tested. [KSCN]-50 values were defined as the concentration of thiocyanate required to decrease the amount of antibody bound to ferritin by 50%.

Lymphocyte marker analysis.

Approximately 1 million cells were used/test. The cells were harvested at the indicated times after the initiation of priming, washed 1 time in growth medium and resuspended in 1 ml cold PBS + % BSA + 0.02 M. sodium azide (washing buffer). Lymphocytes were incubated for 30 minutes at 4 degrees C with murine monoclonal antibodies reactive with human heavy chain mu, heavy chain gamma, PCA-1, B1, CD3, CD4, CD8, or CD25. The cells were washed 3 times and resuspended in washing buffer with fluorescein isothiocyanate (FITC) - labeled affinity isolated, human absorbed goat F(ab')2 anti-murine IgG (TAGO, Burlingame, CA) for 30 minutes at 4 degrees C. The cells were then washed 4 times and resuspended in 0.4 ml PBS + 1% para-formaldehyde. Quantitation of fluorescence for all samples was done on the same day with an EPICS profile flow cytometer. Dead cells were excluded by forward and 90 degree light scatter measures. Sensitivity and amplification of the signal was set so that recorded labeling of the fluorescence control sample (cells labeled with nonspecific mouse

immunoglobulin as the primary antibody) was 5%. The data presented were calculated by subtraction of the background values of cells labeled with non-specific mouse Ig from the values recorded with samples reacted with cell surface specific antibodies.

B. Results

Comparison of immune responses by single and allogeneic, mixed cultures.

Antigen-dependent immune responses were measured by ELISA as the difference in antigen reactivity of polyclonal antibody in supernatants from cultures primed with antigen and supernatants from control cultures primed with antigen and supernatants from control cultures not exposed to antigen. Co-culturing of lymphocytes prepared from two unrelated spleens provided one of the best methods for support and for detection of antigen-induced production of antigen-reactive immunoglobulin. Lymphocytes from 8 spleens were examined individually and in combination. Low IgM responses were observed when certain spleen preparations were cultured individually, as shown in Figure 1 for spleen A and spleen B. In contrast, some spleen preparations were unresponsive under most conditions tested; for example, spleen D in Table I. However, when cells from 2 spleens, responsive or unresponsive, were co-cultured, a ferritin-dependent IgM response was consistently observed (Figure 1; Table I). Non-specific binding activity, estimated from cultures not exposed to antigen, was also increased under allogeneic culture conditions (Figure 1). However, in spite of increased reactivity of non-immunized cultures, allogeneic stimulation in the presence of antigen always led to an antigen-induced signal greater than the signal expected from the sum of the observed contributions of each spleen alone.

Calculated and observed responses are shown in Table I for all combinations of spleens A, B, C, And D. In general, a greater antigen-induced signal was observed with two spleens than with cultures of more than two or with only one spleen. It was therefore concluded that use of two spleens provided an optimal balance between activation insufficient to induce detectable specific responses and allogeneic activation so intense that low quality, non-specific antibody responses overwhelmed antigen-driven reactions.

Ferritin-dependent production of ferritin-reactive antibody by irradiated single and allogeneic mixed spleen cultures.

Non-immunized syngeneic cultures prepared from either spleen 1 or spleen 2 (Figure 2) produced negligible levels of antibody which cross-reacted with ferritin. After priming with ferritin, induction of low levels of ferritin-reactive antibody was observed. Co-culture resulted in increased non-specific and specific reactivity. Irradiation of single and allogeneic mixed cultures abrogated all responses. However, irradiation of only one component of the mixed culture prior to antigen priming resulted in no loss of specific reactivity. In the case of irradiation of spleen 2, specific induction by antigen was increased approximately two-fold. These results indicated that successful allogeneic stimulation required only one partner with functional B cells, that either spleen in an allogeneic culture could be stimulated by antigen to produce specific antibody, and that B cells from spleen 2 of the mixture shown in Figure 2 produce more non-specific antibody cross-reactive with ferritin than B cells from spleen 1.

Time requirements for antigen priming and for detection of antigen-induced, antigen-reactive antibody.

Co-cultures of lymphocytes from 2 spleens were exposed to 4 concentrations of horse ferritin, from 0 to 1.0 ug/ml, for 1, 2, 3, or 4 days to determine the optimal time required for initial exposure to antigen (priming). After priming, cells were cultured in horse ferritin-free medium to determine the optimal time required for secretion of detectable ferritin-reactive antibody (Figure 3). Cultures primed for 1 day required the longest antigen-free incubation, 3-5 days, to detect even low levels of secreted ferritin-reactive antibody. Furthermore, cultures primed for one day were not sensitive to the lowest concentration of ferritin tested, 0.1 ug/ml. Cells primed for 2 days began to produce detectable ferritin-reactive antibody after 2-3 days incubation and were sensitive to 0.1 ug/ml ferritin, especially when incubated for 5 days after priming. Cultures primed for 3 and 4 days produced detectable ferritin-reactive antibody after a one day incubation in horse ferritin-free medium.

In general, the longer the priming, the sooner ferritin-induced, ferritin-reactive antibody became detectable after removal of antigen. If cultures were primed for 3-4 days and allowed to secrete antibody in the absence of ferritin for more than 2 days, the higher the background reactivity, even when only two spleen cultures were used, prevented discrimination between specific induction by antigen and excessive, non-specific B cell activation. The most consistent ferritin-dependent signals were observed when 3 days were allowed for priming and 2 days for secretion, or when 4 days were allowed for priming and 1 day for secretion. Three days of

priming followed by two days of secretion in the absence of antigen constituted the immunization and detection conditions used for subsequent experiments.

After priming of allogeneic cultures for 3 days, ferritin-reactive antibody production continued for longer than 1 week. In the experiment shown in Figure 5, the culture medium was changed and antigen-reactive antibody produced during each 2-3 day interval following priming was measured. The highest level of production occurred on days 5-7, followed by a steady decline in specific and non-specific reactivity. By days 11-13, antigen-induced responses were barely detectable, and by days 13-16, no ferritin-reactivity was observed. The ratio of ferritin-induced to non-specific reactivity was fairly constant, with a slight optimum in specificity observed on days 3-5.

Antigen-induced, antigen-reactive IgG secretion. Culture supernatants were monitored for antigen-induced IgG as well as IgM secretion. Ferritin-induced, ferritin-reactive IgG responses were of lower frequency than antigen-reactive IgM class responses and their detection required a more sensitive ELISA than used for IgM responses. Under the culture conditions used, IgG responses were observed in 10-30% of the experiments. This low frequency of antigen-induced IgG antibodies is characteristic of a primary response; however, other factors may be involved. When monoclonal antibodies were produced after fusion of in vitro immunized lymphocytes, effective numbers of IgG class monoclonals were always obtained even if a polyclonal IgG response was not observed.

IgG responses were more consistently observed with some spleens and spleen combinations than with others, but cell marker analysis of cells from different spleens revealed no differences which might support IgG secretion by one spleen and not by another. When IgG responses were observed, their expression was consistent within one experiment. As seen in Figure 4, the kinetics of appearance of ferritin-induced, ferritin-reactive IgG antibody paralleled the expression of IgM responses By days 9-11, however, non-specific IgG reactivity was not observed and detection of ferritin-reactive IgG was completely dependent upon exposure to ferritin. Prolongation of either IgM or IgG responses by allogeneic cultures significantly beyond 10 days was neither observed nor expected, since the cellular death rate from cytotoxic killing by allogeneic cells increased significantly by this time.

Analysis of relative affinity/avidity properties of polyclonal, ferritin reactive antibody produced under different conditions.

Quantitation of ease of antibody elution from antigen bound to ELISA plates by exposure to thiocyanate has been used to evaluate relative strengths of binding of different antibody preparations. This method was applied to determine if ferritin-induced, ferritin-reactive antibody produced in vitro could be qualitatively distinguished from the non-specific, ferritin-reactive immunoglobulin detected in non-immunized cultures. The ease of elution of polyclonal antibody from solid phase bound ferritin was evaluated using supernatants from ferritin-primed and from non-immunized, control cultures. As seen in Figure 5, antibody from primed cultures required higher levels of thiocyanate for elution than

supernatants from non-immunized cultures. The molar concentrations of thiocyanate required to elute 50% of the binding activity ([KSCN]-50) produced by non-immunized and primed cultures, respectively, were 2.0 M and 2.6 M.

This method was also used to evaluate the binding properties of ferritin-induced antibodies generated under different culture conditions. If production of high affinity antibodies is desired, evaluation of relative quality without regard for the quality of the response may be misleading. Analysis by thiocyanate elution of ferritin-binding strengths of polyclonal antibody mixtures produced under different culture conditions revealed a significant dependence of antibody binding strengths on immunization conditions. Further, this dependence could not have been predicted from the magnitude of the ferritin-induced responses revealed by ELISA analysis above. [KSCN]-50 values of duplicate cultures primed with or without antigen under different conditions are shown in Table II. Although single cultures of cells from a responsive spleen B, produced ferritin-induced, ferritin-reactive IgM, [KSCN]-50 values were similar for immunized and control cultures. In contrast, co-cultures of spleens A + B primed with ferritin in medium containing 10% FCS produced antibody with significantly higher [KSCN]-50 values than antibody produced by non-immunized cultures. Differences between immunized and control cultures reached a peak for antibody produced days 7-9, but became insignificant with antibody produced after day 9.

Exposure of allogeneic cultures to antigen for 10-20 hours in the absence of serum usually increased the level of ferritin-induced, ferritin-reactive antibody measured by ELISA, because background activity of

non-immunized cultures was lower. However, differences in [KSCN]-50 values between immunized and control cultures were not as large nor as consistent when FCS was absent during initial priming as when cultures were continuously supplemented with FCS (Table II). These results illustrate the importance of achieving a balance among different forms of activation for induction of antibodies of the desired quality as well as quantity.

Effects of non-lymphokine factors on antigen-induced, antigen-reactive Ig secretion and on total Ig secretion.

Effects of the non-lymphokine factors, muramyl dipeptide (MDP) and pokeweed mitogen (PWM), on ferritin-induced, ferritin-specific responses were tested using both single and allogeneic mixed cultures (Table III). PWM stimulated overall Ig secretion, but did not enhance specific induction by antigen. MDP also stimulated Ig secretion in general and, in the experiment shown in Table III, enhanced the effects of exposure to antigen. However, specific stimulation by MDP was not consistent, and was usually observed only under sub-optimal culture conditions (data not presented). These results further indicated that allogeneic two-spleen cultures provided sufficient stimulation for induction of antigen-specific responses, and that additional non-specific mitogenic stimulation was either inhibitory or was not usually required.

Influence of adherent cells on the in vitro response to horse ferritin.

An initial protocol for establishment of immunization cultures included overnight incubation in large flasks to allow recovery from thawing prior to stimulation with antigen. This step resulted in

depletion of adherent cells. Comparison of depleted and non-depleted cultures revealed that non-depleted cultures responded better to horse ferritin than depleted culture (Figure 6). Spleen 2 alone was not significantly responsive under either condition but spleen 1, which was unresponsive in depleted cultures, gave a limited response to low concentrations of antigen and a large response to high levels when adherent cells were not depleted. In mixed cultures of spleens 1 and 2, reactivity of non-primed cells was reduced and sensitivity to low levels of antigen was increased when adherent cells were not depleted. These results demonstrate that antigen may be presented more efficiently when immunizations are carried out in the presence of optimal numbers of adherent cells.

In vitro human immune responses to human ferritin.

Responses to human ferritin were also tested to determine if in vitro human immune responses would be detected to human as well as to foreign antigens. Responses to human ferritin were not observed in adherent cell depleted cultures under conditions which supported consistent responses to horse ferritin (Figures 6C, 7). However, when freshly thawed spleen cells were not preincubated overnight, the cells not only responded better to horse ferritin, they also now responded to human ferritin (Figures 6D, 7). When depleted cultures were primed for 2 days with human ferritin and then incubated for a secretion period of 5 days instead of for 3 days, a small response to human ferritin was observed. If depleted cultures were reconstituted with adherent cells at the time of priming, a response to human ferritin was then again observed after secretion for 2 days. Human ferritin-induced IgG responses by non-

depleted cultures were also observed in some experiments (Figure 7A). Specific IgG responses were lower, but paralleled IgM responses.

In vitro antigen priming with foreign immunoglobulins.

In order to assess the applicability of in vitro immunization conditions developed for ferritin to other protein antigens, reactivity to priming with murine monoclonal IgG antibodies was tested under conditions which supported responses to horse and to human ferritin. Antigen-induced, antigen-reactive IgM antibody responses to two monoclonal preparations were detected (Figure 8). In general, background reactivity of non-immunized supernatants was lower when assayed on a monoclonal murine immunoglobulin than on ferritin. Polyclonal, antigen-dependent IgG reactivity was not observed with these antigens, but fusion of lymphocytes primed with a murine monoclonal antibody produced antigen-specific IgG as well as IgM class monoclonal antibodies.

Cellular aggregation

10-20 hours after initiation of priming many cell aggregates appeared, which were visible to the naked eye from the bottom of the culture dishes. If disaggregated by vigorous aspiration, the aggregates began reforming within several hours. Their size depended upon the presence, concentration and the nature of the antigen, on cell density, and on the presence of allogeneic lymphocytes. Antigen-free cultures formed aggregates which were barely detectable by the naked eye. Exposure to horse ferritin induced larger aggregates than human ferritin. Less complex antigens of lower molecular weight, such as the murine immunoglobulins, induced aggregates barely larger than those seen with non-

primed cultures. Larger aggregates were also induced in allogeneic than in single cultures. Cellular aggregation also depended upon the presence of FCS. Supplementation with FCS could be delayed during priming for 15-20 hours without reduction of antigen-dependent responses, but macroscopic aggregates did not begin to form until several hours after addition of FCS.

Cell marker analysis.

Cell marker analysis indicated that human splenocytes could differentiate to a limited extent when cultured under appropriate conditions. The following cell surface markers were analyzed using spleens B + D at day 0, 3, 5, 7 and 10 after initiation of priming: IgG, IgM, B1, PCA-1, T3, T4, T8, and the IL-2 receptor (CD25). Spleens B and D did not differ significantly except that spleen D preparations contained 17% fewer surface IgM bearing cells than spleen B. Changes observed with allogeneic cultures as a function of time were approximately 50% decreases in the percentages of cells bearing surface IgG, IgM, and B1 (Table IV). The percentage of T8 positive cells increased slightly, whereas T3 positive cells were relatively unchanged and T4 positive cells decreased slightly. Striking time-dependent changes included a sharp, approximately ten-fold increase in the percent of IL-2 receptor bearing cells beginning before day 3, followed by an approximate two-fold increase in the percent of PCA-1 positive cells between days 5 and 7, which paralleled the decrease of B1 bearing cells. Cell surface changes were not significantly influenced by ferritin; similar but less striking changes were observed with single cultures.

Monoclonal antibodies produced against horse ferritin.

Allogeneic cultures of human lymphocytes were primed with horse spleen ferritin for 1, 2, or 5 days and then fused (Table I). Two separate but identical fusions, 4 and 5, were performed with identical 5 day-primed cultures to estimate fusion-to-fusion reproducibility. An additional set of cells was maintained in culture for one day but was not intentionally exposed to horse ferritin (fusion 1). Fusion frequency, Ig secretion, antigen-reactive monoclonal antibody production, and antigen-specific monoclonal antibody production were monitored for each fusion.

Fusion frequencies were similar for cells primed with ferritin for 1 through 5 days (35-50 clones/million lymphocytes), but non-immunized cells fused at a lower frequency (17 clones/million lymphocytes) than primed cells. Non-immunized cells appeared less activated, forming smaller macroscopic aggregates than primed cells. The number of cells in ferritin-primed cultures did not significantly increase relative to control cultures.

A large percentage of the hybridoma clones produced in these fusions secreted immunoglobulin (20-60%). In the set of fusions described in Table I, IgG-secreting clones were 5-10 times more numerous than IgM-secreting clones. The majority of the fusions in our laboratory have produced approximately equal numbers of IgG and IgM secreting hybrids (see Table II and III).

In general, the patterns of induction of ferritin-specific and ferritin-reactive monoclonal antibodies were similar (see Figure 9 for comparison of binding

specificities of ferritin-reactive versus ferritin-specific monoclonal antibodies). Exposure to ferritin for only one day did not significantly increase the percent of ferritin-reactive or of ferritin-specific hybridomas compared with those produced from the non-immunized, control cultures. A greater number of ferritin-reactive hybrids were produced from cells immunized for one day, but fusion frequencies were greater. After 2 days of priming, the percentage of ferritin-reactive clones rose from 5-6% to 16%. After 5 days, the level dropped to 10-12%, but was still significantly higher than the background observed with cells cultured for one day. Cells exposed to ferritin for as long as 8 or 15 days fused with similarly high frequencies. However, out of 93 tested, none secreted stable, ferritin-reactive monoclonal antibody.

All antibody-secreting clones which initially reacted with ferritin by ELISA were tested further for ferritin specificity. Although many of the monoclonal antibodies initially identified cross-reacted with other proteins (Figure 9), and would not ordinarily be of interest, we have monitored their incidence to determine if certain conditions or antigens induced higher percentages of specific antibodies than others. No such culture conditions have been identified, but allogeneic antigens induced a higher percentage of specific antibodies than horse ferritin (Table VIII).

Monoclonal antibodies produced against human ferritin.

A similar series of fusions used lymphocytes immunized with human instead of with horse ferritin (Table VI). Fusions 1-3 used lymphocytes maintained in culture for 2 days. Fusion 1 used lymphocytes not

intentionally exposed to ferritin, while fusions 2 and 3 were primed with 0.25 ug/ml ferritin. Fusions 2 and 3 were identical, but were maintained separately to assess fusion-to-fusion variation. Fusions 5 and 6 were performed on days 4 and 6, respectively, after initiation of priming with 2.5 ug/ml human ferritin instead of with 0.25 ug/ml.

The fusion frequency of cells primed for 2 days with 0.25 ug/ml human ferritin was higher than the fusion frequency of control non-immunized cells, but was not as high as cells immunized with 0.25 ug/ml horse ferritin. Cellular aggregates were also smaller when cultures were primed with human than with horse ferritin. Cultures immunized for 4 or 6 days with a 10-fold higher level of human ferritin fused with frequencies equivalent to cultures primed with 0.25 ug/ml horse ferritin. Since fusion frequencies of horse ferritin primed cells were not influenced by time of priming, the higher frequencies of fusions 4 and 5, relative to fusions 2 and 3, may have resulted from the higher concentration of human ferritin used, although fusion-to-fusion variation cannot be excluded.

In these fusions, 30-45% of the hybrids produced by lymphocytes maintained in culture for 2-4 days secreted immunoglobulin. Ferritin did not influence the number of Ig secreting hybrids produced. However, comparison of fusions 4 and 5 revealed that cells primed for 6 days produced far fewer secreting clones than cells primed for 4 days (8% versus 44%). This result is similar to the low productivity of fusions of cells immunized with horse ferritin for 8 and 15 days. According to cell marker analysis, the percent of B1+ cells decreased and the percent of plasma (PCA-1+) cells increased two-fold between days

5 and 7. These results suggested that Ig-secreting hybrids were more likely to be produced from B cells fused before rather than after differentiation to plasma cells, and that the most productive fusions resulted from cells immunized for more than one but for no more than five days.

Monoclonal antibodies produced against a murine monoclonal immunoglobulin.

Human in vitro immune responses to murine monoclonal antibodies were tested in efforts to produce human anti-idiotypic monoclonal antibodies to murine monoclonal antibodies. Allogeneic cultures of human lymphocytes were primed with 2 ug/ml of a murine monoclonal antibody for 2, 3, or 4 days (Table VII, fusions 2, 3, 4, and 5). Cells used for fusion 1 were cultured for 2 days but were not primed with specific antigen. Fusions 3 and 4 were identical except that fusion 4 cells were cultured at 1.5 million cells/ml instead of at the standard 3 million cells/ml. Similar fusion frequencies were observed with each of the immunizations. Non-primed cells were again less fusogenic. Of the antigen-reactive clones identified, 63% were antigen-specific. 92% of antigen-reactive IgG clones were antigen-specific, whereas only 50% of the IgM antigen-reactive clones were specific. This result is in agreement with the predicted greater specificity of IgG antibodies.

Fusion summary

The results of fusions of lymphocytes immunized with either horse ferritin, human ferritin, or with a murine monoclonal IgG immunoglobulin are summarized in Table VIII. Of the hybrids produced from cells immunized with either of the foreign proteins, 9-10% were antigen-reactive. Of the clones produced from cells immunized with human ferritin, only 3% were

antigen-reactive, a result which might be expected from the lower immunogenicity of the human protein. Of the antigen-reactive hybrids produced from cells primed with horse ferritin, 29% were highly antigen specific. Relative to the number of antigen-reactive hybridoma, antigen specificity was much higher for monoclonal antibodies produced from cells primed with either human ferritin (65%) or with the murine IgG molecule (63%). The greater apparent lymphocyte activation by horse ferritin than by human ferritin or by the murine IgG may be related to the greater production of cross-reactive antibodies from horse ferritin-primed cells. Comparison of antigen-specific IgM versus IgG production showed that the highest ratio of IgM:IgG antibodies resulted from immunizations with the allogeneic, human ferritin. Immunizations with xenogeneic antigens showed less bias towards production of IgM class antibodies.

Stability of human monoclonal antibody production.

Ferritin-reactive monoclonal antibody production was initially monitored with hybrids growing in 96 well plates. When a hybrid clone was first identified as secreting ferritin-reactive antibody, it was expanded to a 48 and then to a 24 well plate, for a minimum number of 3 passages. The presence of ferritin-reactive monoclonal antibody was monitored at each passage. Approximately 50% of the clones initially positive for ferritin-reactive antibody secretion lost production within 3 passages (Table IX). Hybrids used for further study, as well as those used to compile the data presented in Tables V-VII, were selected from those which were stable beyond 3 passages. Most of these selected clones remained stable for at least several months. Unstable hybrids could therefore be eliminated early in the procedure,

with the remaining hybrids having a high probability (>90%) of maintaining antibody production.

Quantitation of anti-ferritin IgG secretion.

Antibody concentrations of supernatants from confluent 24 well terminal cultures, secreting ferritin-reactive IgG antibody, were measured by quantitative ELISA. Levels of immunoglobulin secreted under these conditions ranged from 1 to 50 ug/ml (Table X). The scale-up of two relatively high affinity anti-ferritin antibodies to spinner flasks resulted in levels of production from 0.5-2.0 ug/ml. These hybrid cells often grew faster in spinner culture than in flasks, but antibody production levels were lower. Preliminary results indicate that growth of cells in a coreactor (Synbiotics, Incorporated, San Diego) leads to 5-10 times higher antibody concentrations than growth in spinner culture.

Characterization of ferritin-specific monoclonal antibodies.

IgG class human monoclonal antibodies were purified in one step by affinity chromatography on Protein G. SDS-polyacrylamide gel electrophoresis revealed heavy and light chain bands. Western blotting using anti-human heavy and light chain and anti-mouse heavy and light chain reagents confirmed that the monoclonal antibodies tested, products of a human lymphocyte fused with a human X mouse heteromyeloma, were of human and not of murine origin.

Light chain analysis of 16 IgM class antibodies revealed 6 with a lambda light chain, 9 with a kappa light chain, and 1 with lambda + kappa reactivity. Analysis of 95 IgG class antibodies showed 61 with a

lambda light chain, 31 with a kappa light chain, and 3 with lambda + kappa light chains. Since hybrids had not been subcloned at this stage, some of the cultures were not monoclonal.

Affinity measurements by competition ELISA, using two subcloned, purified highly specific anti-ferritin IgG antibodies revealed dissociation constants in the range of 1-2 X 10(-8) M (Figure 10). Competition assays utilized horse ferritin, the monoclonal antibody 14-2-2-59 was raised against horse ferritin and antibody 21-1B-9 was made against human ferritin. Of the IgM supernatants tested for ferritin specificity, 13-5-3-18 was the most specific and most highly reactive. This monoclonal antibody was tested for reactivity with ferritin-containing tissues by immunohistological analysis. Figure 11 shows reactivity 13-5-3-18 with human liver tissue in comparison with reactivity of a preparation of polyclonal human IgM antibodies used as a negative control. A second control, consisting of a human IgM monoclonal antibody produced by the same procedure as 13-5-3-18 but produced against a different antigen, was also negative.

C. Discussion

The present work focuses on conditions that will support primary immunization reactions in vitro. The results were achieved without Ts cell depletion or adjustment of T:B cell ratios, and suggest that an initial level of 30-40% Ts cells in the presence of 15-25% Th cells, 40-60% B cells (Table IV), and more than minimal levels of adherent cells will support antigen priming when lymphokines are generated by allogeneic stimulation. These cell numbers result in a B:T cell ratio of approximately 1:1.

Supplementation with adherent cells or with factors

secreted by adherent cells, such as IL-1, has been another variable requirement. The present protocols using spleen or tonsil preparations did not specifically require supplementation with either adherent cells or monocyte-secreted factors. The present finding that immunizations with ferritin, especially with human ferritin, were better when adherent cells were retained, indicate that these cells and/or their products are helpful (horse ferritin) or are an absolute requirement (human ferritin) for efficient priming with T-cell dependent, protein antigens.

A minimal level of lymphokine stimulation is required for successful in vitro priming and it is likely that the appropriate stimulation can be achieved for most tissue preparations in a variety of ways. The method of generation of lymphokines may not be nearly as important as the achievement of optimal levels at the appropriate times. For spleen tissue prepared according to the present protocol, syngeneic culture without supplementation was suboptimal for certain spleens and not at all effective for others. However, allogeneic culture of two spleens appeared sufficient for any combination of two spleens tested, while culture of more than two appeared to lead to excessive non-specific stimulation, similar to effects observed when PWM was included in the immunization cultures.

Another potentially critical factor in the support of antigen priming by lymphokines is the timing of exposure of the cells to individual factors. According to cell marker studies (Table IV), many cells differentiate in culture as the response proceeds. The response to a lymphokine by a cell in one state may differ significantly from its response

during or after differentiation. In the present experiments, addition of exogenously generated lymphokines in the form of a supernatant (MLCS) or endogenous generation by non-specific mitogenic stimulation of T cells were less effective than allogeneic co-culturing. These results suggest that the levels and kinetics of lymphokine exposure generated by allogeneic stimulation during priming may most closely mimic exposure during in vivo immunization responses.

Post-immunization culture supernatants by ELISA analysis revealed low, but detectable IgG responses in 10-30% of our experiments when cells were primed with ferritin, but in none of the experiments when cells were primed with murine immunoglobulin proteins. However, fusion of lymphocytes immunized with either antigen led to the production of antigen-specific IgG as well as IgM monoclonal antibodies. The highest numbers of antigen-reactive IgG monoclonal antibodies were derived from fusions of lymphocytes immunized with horse ferritin, for which polyclonal antigen-induced IgG responses were most easily detected prior to fusion. The early appearance of the antigen-driven IgG response in experiments where observed, was surprising.

Polyclonal IgM antibody produced after antigen priming under appropriate conditions (Table II) bound significantly better to ferritin than polyclonal antibody produced from non-immunized cultures. Less complex antigens than ferritin showed lower levels of non-specific binding. Results of the thiocyanate analysis, however, did indicate that ferritin-induced maturation of the anti-ferritin immune response occurred in cultures primed under appropriate conditions. The maturation of the IgM response

observed was probably primarily due to specific binding of ferritin to naive B cells, activation, and their subsequent maturation to antibody-secreting plasma cells and possibly also to memory cells. Production of antigen-specific IgG monoclonal antibodies from fusion of *in vitro* immunized lymphocytes suggests that antigen-driven class switch and affinity maturation might occur *in vitro*, if the IgG secreting hybrids were not generated from cross-reactive hybridoma clones.

The present results demonstrate that *in vitro* activated human lymphocytes can be efficiently fused with a mouse:human heteromyeloma to produce a high percentage of IgG and IgM secreting heterohybridoma cell lines.

The present invention describes specifically the fusion of the K6H6/B5 cell line, developed by Carroll *et al*. *J. Immuno. Methods* 89, 61 (1986), to human splenocytes cultured *in vitro* for 1-6 days. The average fusion frequency was 35 and the range for 15 separate fusions was 17-50 hybrid containing wells/million lymphocytes. *In vitro* culture of lymphocytes, especially if combined with allogeneic stimulation, leads to a highly fusogenic state of activation. The larger cellular aggregates and the higher fusion frequencies observed for immunized relative to the control cultures (Tables V-VII) suggested that fusion efficiency could be influenced by the degree or by the nature of lymphocyte activation.

The level of Ig production was fairly consistent for each hybrid but the range for 24 hybrids varied by 50-fold. Production by some of these cell lines compared well with other human hybrids and with

murine monoclonal antibody production in culture. Ig production was unstable in approximately 50% of the hybrids produced, but the large numbers obtained permitted early elimination of unstable hybrids, leaving a large selection for evaluation of secretion levels, specificity and affinity. Preliminary karyotypic analysis with G-banding of the K6H6/B5 fusion partner revealed an average chromosome number of 91 (range = 77 to 97), surprisingly few identifiable mouse or human chromosomes, and many structures which appeared to be chimeric mouse:human chromosomes. Fusion of this genetic background with human lymphocytes may allow better retention of human chromosomes than fusion with B cells of murine lineage, but the initial instability of secretion in 50% of the hybrids was understandable in view of the karyotypic complexity and abnormality of the K6H6/B5 partner.

The results also revealed that many antigen-specific IgG secreting hybrids could be produced from fusions of in vitro immunized lymphocytes. The IgG-secreting clones observed in these experiments may have developed from in vitro primary responses, since activated lymphocytes can give rise to IgM-secreting hybrids or can undergo class switch to become IgG-secreting plasma cells as part of the primary response. Fusion of either type of cell at an as yet undetermined point or points during their development could, therefore, produce either IgM or IgG-secreting hybrid cells. Possibly, the allogeneic stimulation induced by co-culture of splenocytes from different individuals provided more support for class switching than has previously been observed in vitro.

An alternative explanation for the recovery of antigen-specific IgG class antibodies from the

fusions involves the possibility that such hybrids arose from secondary stimulation of memory cells. Ig receptors on certain memory cells in the splenocyte preparations may recognize determinants on the priming antigens similar enough to previously encountered determinants to lead to activation.

The nature of the antigen used for priming influenced the percent of antigen-reactive hybrids derived/total hybrids produced. The foreign proteins, horse ferritin and mouse IgG, induced 9-10% antigen-reactive clones/total hybrids, while the allogeneic human ferritin induced only 3-4% ferritin-reactive clones/total hybrids (Table VIII). The response to human ferritin, although lower than to foreign proteins, indicates that monoclonal antibodies can be produced to highly conserved and even to self-proteins by <u>in vitro</u> immunization. The similarity of the affinity of 21-1B-9, produced against human ferritin, to the affinity of one of the best antibodies produced against horse ferritin, 14-2-2-59 (Figure 11), indicated that <u>in vitro</u> antibodies produced against self could be similar quality to antibodies produced against foreign proteins.

The IgG antibodies selected were affinity purified on Protein G. Immunoblot analysis on ferritin and on extracts of ferritin-containing tissue showed reactivity patterns similar to those observed with a high affinity murine anti-ferritin monoclonal antibody, produced by Hybritech, Inc., reactivity which also requires binding affinities of 10(7)-10(8)/mol. Direct analysis of the strength of antigen binding of two of the purified anti-ferritin IgG antibodies by competition ELISA (Figure 11) also indicated that affinities wherein the range of 10(7)-10(8)/mol. The induction of IgG monoclonal

antibodies having apparent affinities of this strength is compatible with a specific primary response in combination with class switching, but probably not associated with extensive somatic mutation. Alternatively, the antigen-reactive, IgG-secreting hybrids could be products of cross-reactive memory responses, where affinities to primary antigens are high, but affinities to cross-reactive, inducing antigens are usually, but not always, lower.

Table I. Analysis of ferritin-reactive IgM antibody secretion in vitro primed syngeneic and allogeneic lymphocyte cultures: comparison of predicted with observed responses.

Ferritin-dependent Response (Δ OD_{490})		
Spleen	Observed	Calculated
A	.15	--
B	.06	--
C	.14	--
D	ND	--
A+B	.34	.10
A+C	.33	.25
A+D	.13	.08
B+C	.28	.10
B+D	.05	.03
C+D	.18	.07
A+B+C	.21	.12
B+C+D	.16	.07
A+B+D	.15	.10
A+B+C+D	.19	.09

Human splenocytes were prepared without depletion of adherent cells and cultured in the presence or absence of 1 ug/ml horse spleen ferritin as described

above. Observed values represent the difference in OD_{490} values between control and ferritin-primed cultures. Calculated values for mixed cultures were derived from the summation addition of the appropriate fractions of the responses of syngeneic cultures (i.e., calculated value for

$$A+B+C = \frac{1}{3}(.15) + \frac{1}{3}(.06) + \frac{1}{3}.14 = .12.$$

ND = not detected

Table II. Use of affinity/avidity estimations to evaluate different culture conditions for in vitro antigen priming.

Spleen (%)		Serum time (days)	Secretion Control exp 1	Secretion Control exp 2	$[KSCN]_{50}$ (M) Immunized exp 1	$[KSCN]_{50}$ (M) Immunized exp 2
B	10%	3-5	2.0	2.1	1.8	2.3
A+B	10%	3-5	2.0	2.0	2.6	2.6
		5-7	2.6	2.6	2.9	2.8
		7-9	2.2	1.7	2.9	2.9
		9-11	1.5	1.2	2.2	1.4
A+B	0%	3-5	2.1	2.2	2.3	2.3
		5-7	1.8	2.4	2.9	2.5
		7-9	2.1	2.4	2.7	2.4
		9-11	1.5	1.8	1.5	2.0

Human splenocytes were prepared without depletion of adherent cells as described above. Either syngeneic or 1:1 co-cultures of 2 spleens were primed with 0 or 1 ug/ml horse spleen ferritin for 3 days. The

ferritin was removed by washing and supernatants were collected at the indicated times. The ferritin reactivity of the secreted immunoglobulin was analyzed by ELISA and relative affinity/avidity estimations were made as described above. The numbers shown represent the Molar concentrations of KSCN required to decrease the amount of antibody bound to ferritin on the assay plates by 50% ([KSCN]-50).

Table III. Non-specific mitogen effects on (A) secretion of total Ig and (B) on ferritin induction of ferritin-reactive antibody.

	Spleen 1	Spleen 2	Spleen 1 & 2
A. μg/ml total immunoglobulin			
No additions	2.4±0.4	0.6±0.1	12.3±1.5
+ PWM	30.8±3.7	2.6±1.0	20.1±0.8
+ MDP	5.8±1.4	4.0±1.0	21.5±1.8
B. ferritin-induced, ferritin-reactive OD_{490}			
No additions	0	0	0.28±0.14
+ PWM	0	0	0.13±0.08
+ MDP	0.29±0.8	0.04±0.02	0.46±0.09

Table IV. Cell marker analysis of human splenocytes as a function of time in culture.

Marker	Day 0	3		5		7		10	
		1	2	1	2	1	2	1	2
IgG	49	17	16	15	14	9	12	11	8
IgM	30	22	20	17	15	8	10	7	10
B1	51	44	41	37	35	17	15	11	12
PCA-1	14	14	13	17	15	31	24	33	23
T3	37	40	42	45	42	46	36	46	40
T4	20	17	18	17	16	12	9	20	16
T8	34	38	40	47	46	43	44	49	46
IL-2 receptor	3	17	15	32	30	35	35	24	22

Human splenocytes were prepared without depletion of adherent cells and cultured as discussed above. Parallel experiments were performed for each surface marker on days 3, 5, 7, and 10 and depicted on the table as 1 or 2. Cultures were harvested analyzed as described above.

Table V. Hybridoma clone production from human lymphocytes immunized <u>in vitro</u> with horse spleen ferritin.

Fusion	1	2	3	4	5
Antigen (.25 ug/ml)	-	+	+	+	+
Culture Time (days)	1	1	2	5	5
# cells fused (x 10^6)	5	5	5	5	5
# hybrid-containing wells	86	249	230	177	199
Fusion Frequency	17	50	46	35	40
# Secretors					
IgM	1/59 (2%)	5/173 (3%)	14/137 (10%)	7/88 (8%)	6/90 (7%)
IgG	12/59 (20%)	49/173 (28%)	80/137 (58%)	46/88 (52%)	48/90 (53%)
Reconfirmed ferritin-reactive					
IgM	1	0	4	6	5
IgG	4	10	33	16	14
Ferritin-specific (vs. β-galactosidase)					
IgM	0	0	1	1	0
IgG	2	4	11	5	3

Immunizations, fusions, and hybridoma screening were performed as described above. Lymphocytes were primed with either 0 or 0.25 ug/ml horse spleen ferritin for 1 (fusions 1 and 2), 2 (fusion 3), or 5 (fusions 4 and 5) days. the number of IgM or IgG secreting hybrids is shown as the number of secretors per the number of clones tested for IgM or IgG secretion.

Table VI. Hybridoma clone production from human lymphocytes immunized *in vitro* with human ferritin

Fusion	1	2	3	4	5
Ferritin (ug/ml)	0	.25	.25	2.5	2.5
Culture Time (days)	2	2	2	4	6
# lympho-cytes fused (x 10^6)	3.8	4	4	4	4
# hybrid-containing wells	31	49	64	266	268
Fusion Frequency (clones/10^6 lymphocytes)	8	12	16	32	39
Ig secreting					
IgM	3 (10%)	4 (8%)	12 (9%)	62 (23%)	6 (2%)
IgG	11 (35%)	12 (24%)	19 (30%)	55 (21%)	15 (6%)
Ferritin-reactive					
IgM	0	2	4	8	1
IgG	1	1	1	3	2
Ferritin-specific (vs. β-galactosidase and BSA)					
IgM	-	1	1	8	1
IgG	1	1	0	2	0

Immunizations, fusions and hybridoma screening were performed as described above. Lymphocytes were cultured at 3 x 10^6 cells/ml for 2 (fusions 1-3), 4 (fusion 4), or 6 days (fusion 5) in the presence of 0.25 or 2.5 ug/ml human ferritin as indicated.

Table VII. Hybridoma clone production from human lymphocytes immunized <u>in vitro</u> with murine monoclonal IgG

Fusion	1	2	3	4	5
Culture Time (days)	2	2	3	3	4
Antigen (ug/ml)	0	2	2	2	2
Cell density (x $10^{6/ml}$)	3	3	3	1.5	3
# hybrid-containing wells	27	107	116	98	1.26
Fusion Frequency	7	27	29	25	32
Antigen-reactive					
IgM	1	10	12	5	2
IgG	0	4	8	1	0
Antigen-specific					
IgM	1	5	4	4	1
IgG	0	4	7	1	0

Immunizations, fusions, and hybridoma screening were performed as described above.

Table VIII. Comparison of hybrid production from lymphocytes immunized with horse ferritin, human ferritin, or with murine monoclonal IgG.

Antigen	# Clones reactive	# Antigen-specific	# Antigen-	IgM	IgG
Horse ferritin	941	93	27	2	25
Human ferritin	678	23	15	11	4
Murine monoclonal IgG	474	43	27	15	12

Immunizations, fusions, and hybridoma screening were performed as described above. Lymphocytes were cultured and immunized as described in Tables V-VII.

Table IX. Stability of immunoglobulin production by human hybridomas.

Immunogen	# initial positive clones	# reconfirmed (passaged 3 x)
horse ferritin	192	92 (48%)
human ferritin	40	24 (60%)

Immunizations, fusions, and hybridoma screening were performed as described above.

Table X. Quantitation of immunoglobulin production by IgG secreting human hybridoma clones.

Clone #	Ig (ug/ml)	Ferritin-reactivity (O.D.)
1	50 ± 11	1.15
2	42 ± 9	1.65
3	38 ± 6	.2
4	31 ± 13	.2
5	28 ± 5	.8
6	27 ± 6	.6
7	23 ± 6	.7
8	20 ± 5	.6
9	19 ± 4	1.5
10	17 ± 7	.2
11	14 ± 4	.1
12	12 ± 3	.9
13	12 ± 3	.2
14	10 ± 2	.1
15	9 ± 1	.1
16	7 ± 2	.2
17	6 ± 1	.2
18	6 ± 1	.1
19	5 ± 1	1.3
20	5 ± 1	.1
21	3 ± 1	.2
22	2 ± 1	.1
23	1.4±0.3	.1
24	1.3±0.3	.4

Hybridoma screening and quantitation assays were performed as described above.

Concluding Remarks

The foregoing description details specific methods that can be employed to practice the present invention. Having detailed such specific methods initially used to prepare, isolate, characterize and use the antigen specific high affinity monoclonal antibodies hereof, and a further disclosure as to specific model procedures and entities, the art skilled will well enough know how to devise alternative reliable methods for arriving at the same information and for extending this information to other related preparations of such monoclonal antibodies. Thus, however detailed the foregoing may appear in text, it should not be construed as limiting the overall scope hereof; rather, the ambit of the present invention is to be governed only by the lawful construction of the appended claims.

CLAIMS

1. An in vitro immunization culture comprising lymphoid tissue derived human lymphocytes having essential numbers of autologous accessory cells, immunized with a specific antigen under culturing conditions not requiring supplementation with growth or other factors, such that effective numbers of human monoclonal IgG antibodies having affinities with said specific antigen of at least about 5×10^7 liters/mole are derived from said lymphocytes.

2. A culture according to Claim 1 wherein said lymphoid tissue is human spleen.

3. A culture according to Claim 1 wherein said lymphocytes are human splenocytes.

4. A culture according to Claim 1 wherein said antibodies are produced from in vitro immunization of lymphocytes having multiple macroscopic aggregate forms.

5. A culture according to Claim 4 wherein said lymphocytes are splenocytes.

6. A culture according to Claim 4 that is an allogeneic coculture.

7. A culture according to Claim 4 containing multiple splenoids.

8. A stable continuous cell line that produces antigen specific human monoclonal antibodies defined according to Claim 1.

9. A stable continuous cell line according to Claim 8 wherein said lymphocytes are immortalized via fusion with immortal fusion cell partners.

10. A stable continuous cell line according to Claim 8 prepared via recombinant means and harboring operatively DNA encoding the variable region of said antibody.

11. A stable continuous cell line according to Claim 8 wherein nucleic acid encoding the variable region of said antibody is amplified and identified via application of polymerase chain reaction.

12. A hybridoma according to Claim 9 wherein said immortal fusion cell partners are selected from the group consisting of an immortalized lymphoid, myeloma and heterohybridoma cell line.

13. A hybridoma according to Claim 9 capable of producing antigen specific human monoclonal antibodies having an antigen affinity of at least about 5×10^7 liters/mole.

14. A hybridoma according to Claim 9 wherein said monoclonal antibodies are IgG antibodies.

15. A hybridoma according to Claim 9 that secretes a human monoclonal antibody to a human antigen.

16. A hybridoma according to Claim 9 that secretes a human monoclonal antibody to a non-human antigen.

17. A human monoclonal antibody when prepared from a cell line according to any one of Claims 8, 9, 10, 11, 12, 13 or 14.

18. A human monoclonal antibody according to Claim 17 specific for a human antigen.

19. A human monoclonal IgG antibody according to Claim 18.

20. A process of preparing an antigen-specific human monoclonal antibody comprising culturing in vitro lymphoid tissue derived human lymphocytes having essential numbers of autologous accessory cells, immunized with a specific antigen under conditions not requiring supplementation with growth or other factors, such that effective numbers of human monoclonal IgG antibodies having antigen affinities of at least about 5×10^7 liters/mole are derived from said lymphocytes, means for producing said human monoclonal antibodies from said lymphocytes and recovering said human monoclonal antibodies.

21. A process according to Claim 20 wherein said culturing is of an allogeneic coculture.

22. A process according to Claim 20 wherein said human lymphocytes are prepared and cultured by a) using hypotonic lysis of red blood cells, b) minimizing room temperature incubations to avoid loss of accessory cells, c) retaining small fragments of lymphoid tissue in the final cell suspension prior to freezing, and d) including or supplementing small fragments of lymphoid tissue in the cell suspension.

23. A process according to Claim 20, wherein said means comprises fusing said lymphocytes with immortal fusion cell partners.

24. A process according to Claim 23 wherein said immortal fusion cell partners are selected from the group consisting of an immortalized myeloma, plasmacytoma, heteromyeloma and heterohybridoma cell line.

25. A human antigen-specific human monoclonal antibody having an antigen affinity of at least about 5×10^7 liters/mole when prepared by the process according to Claim 22.

26. An antibody according to Claim 25 that is an IgG antibody.

27. An antibody according to Claim 25 linked with a material capable of modulating cell growth.

28. An antibody according to Claim 27 wherein said material is $yttrium_{90}$.

29. An antibody according to Claim 25 linked with a reporter moiety.

30. An antibody according to Claim 29 wherein said moiety is $indium_{111}$.

31. A method of treating cancer, bacterial or viral disease comprising administering to an individual suffering therefrom an antibody according to Claim 27.

32. A method of testing an individual for presence of disease comprising administering to said individual an antibody according to Claim 29.

33. A human monoclonal antibody that is an antiidiotype of an antibody according to Claim 25.

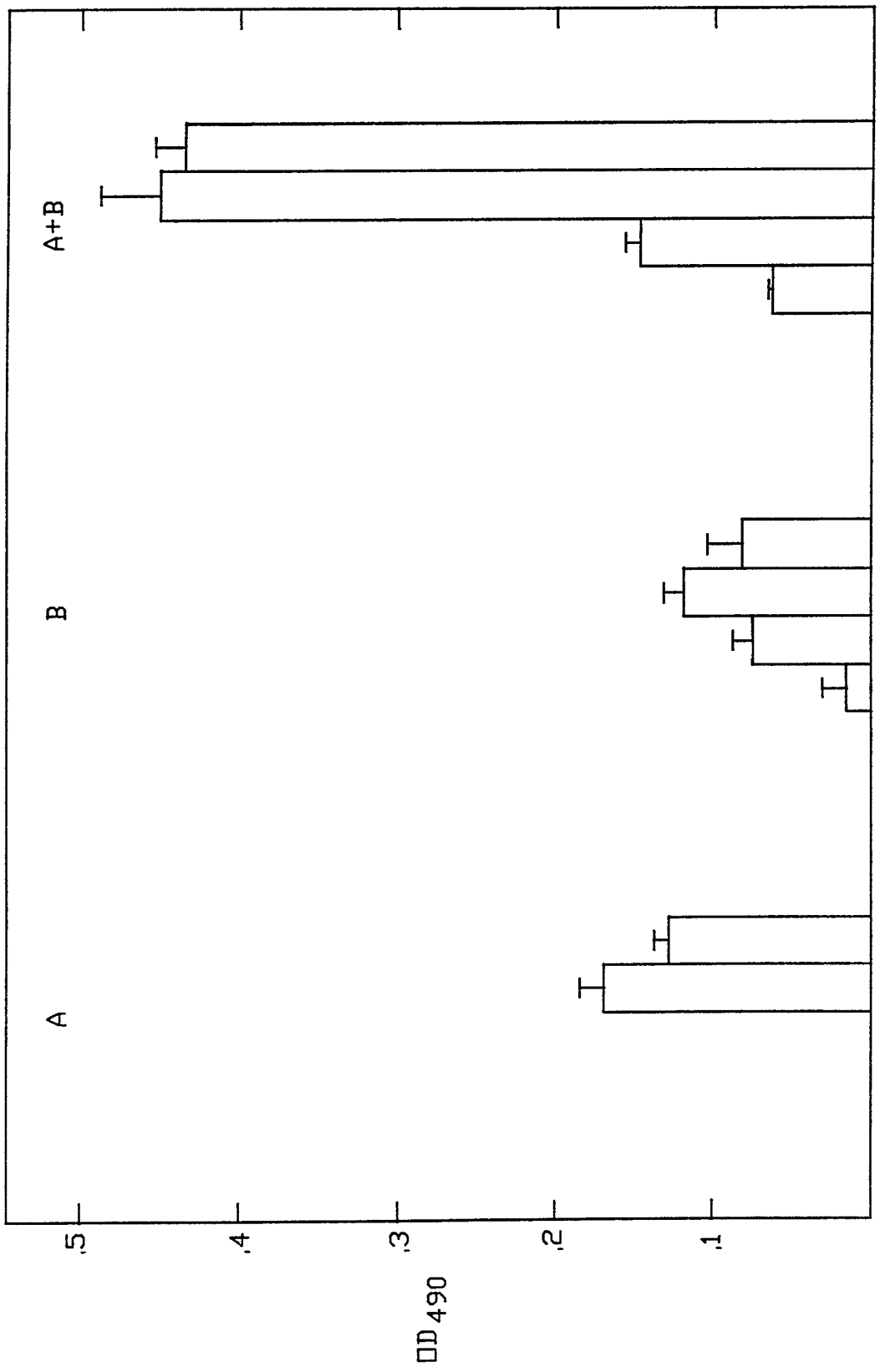

FIG.—1

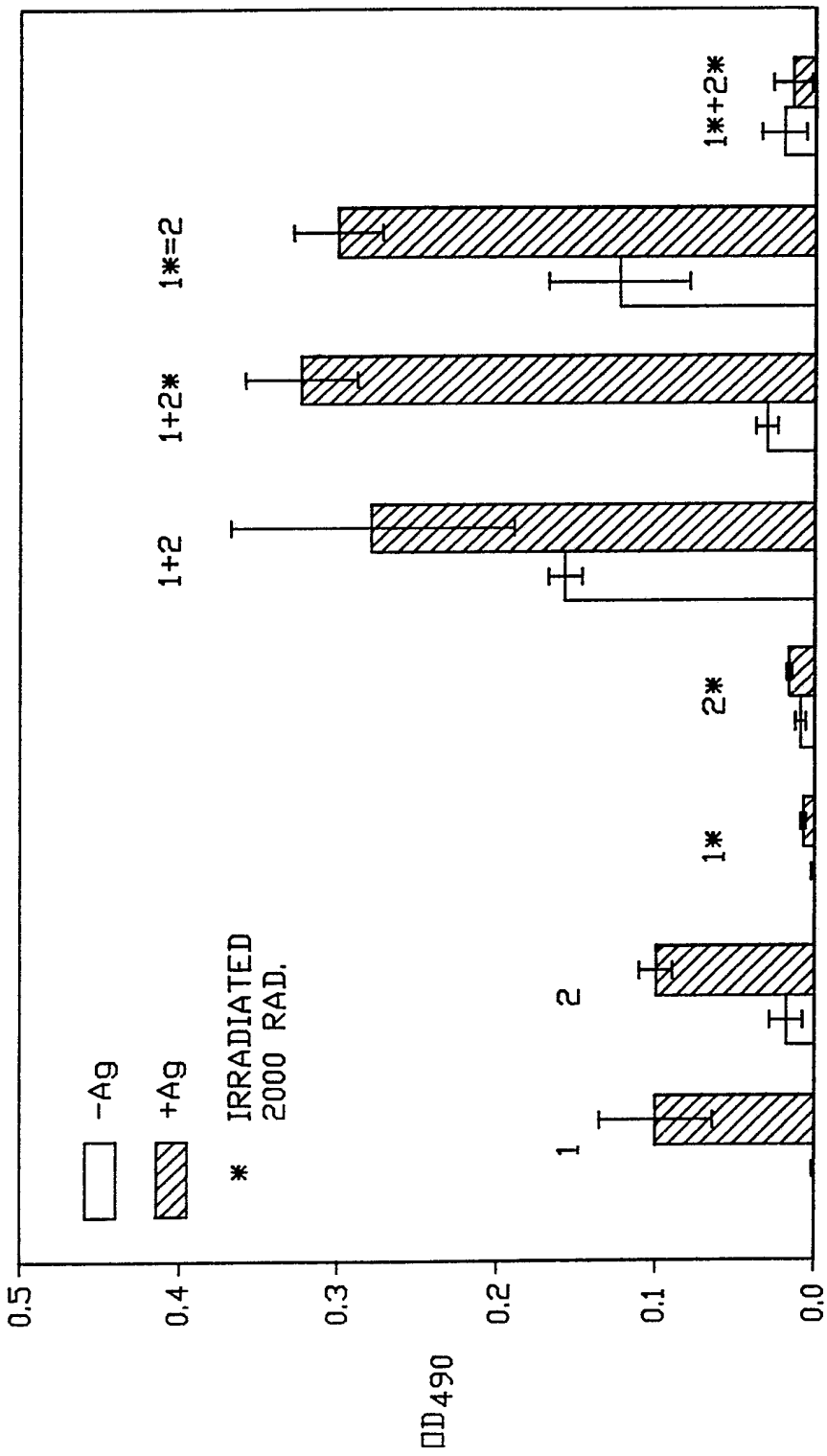
0.5
0.4
0.3
0.2
0.1
0.0
OD490
-Ag
+Ag
* IRRADIATED
2000 RAD.
1
2
1*
2*
1+2
1+2*
1*=2
1*+2*

FIG.-2

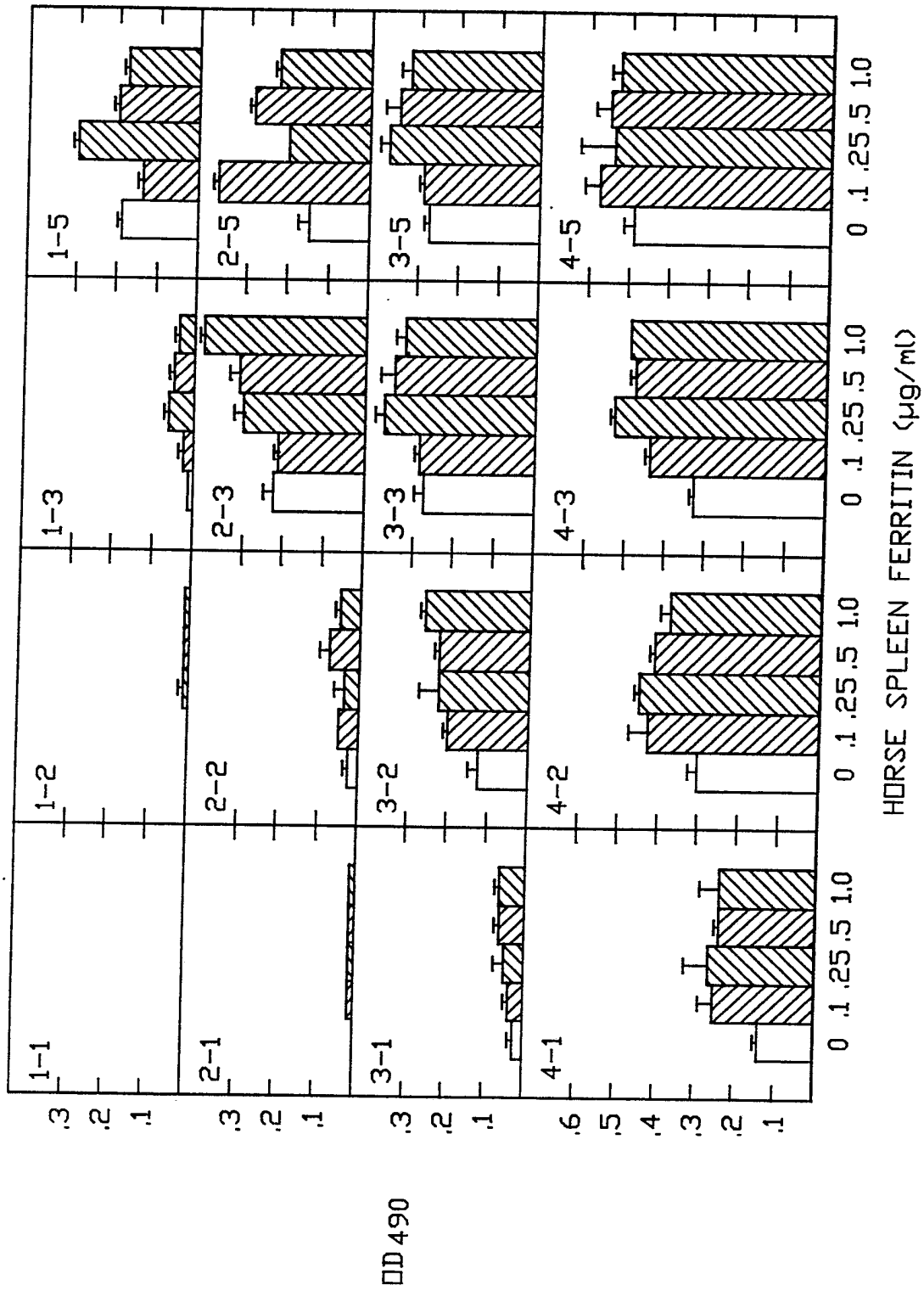
1-1
1-2
1-3
1-5
2-1
2-2
2-3
2-5
3-1
3-2
3-3
3-5
4-1
4-2
4-3
4-5
.1
.2
.3
.4
.5
.6
0 .1 .25 .5 1.0
HORSE SPLEEN FERRITIN (µg/ml)
OD 490

FIG.—3

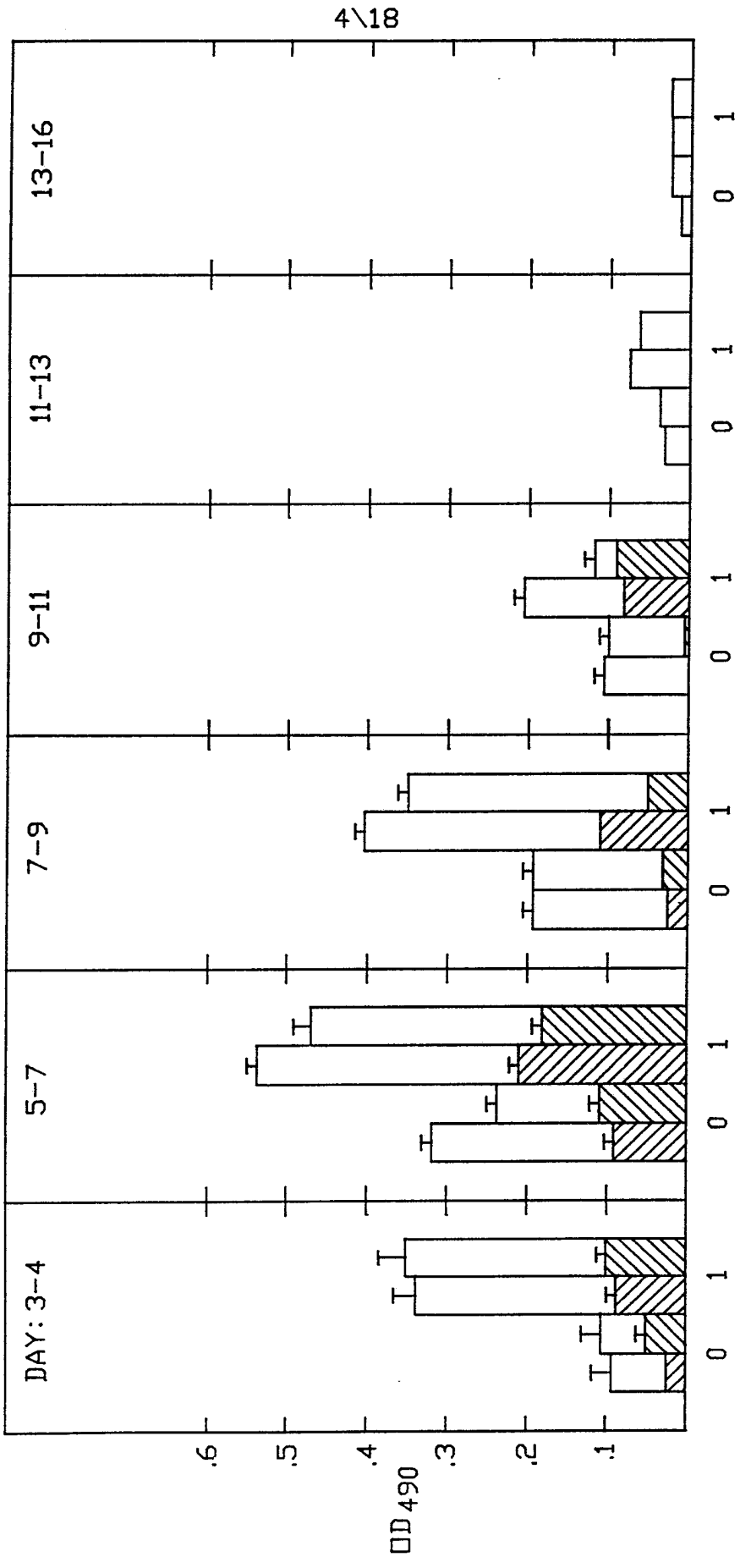
4\18
DAY: 3-4
5-7
7-9
9-11
11-13
13-16
0
1
.1
.2
.3
.4
.5
.6
OD 490
HORSE SPLEEN FERRITIN (µg/ml)

FIG.—4

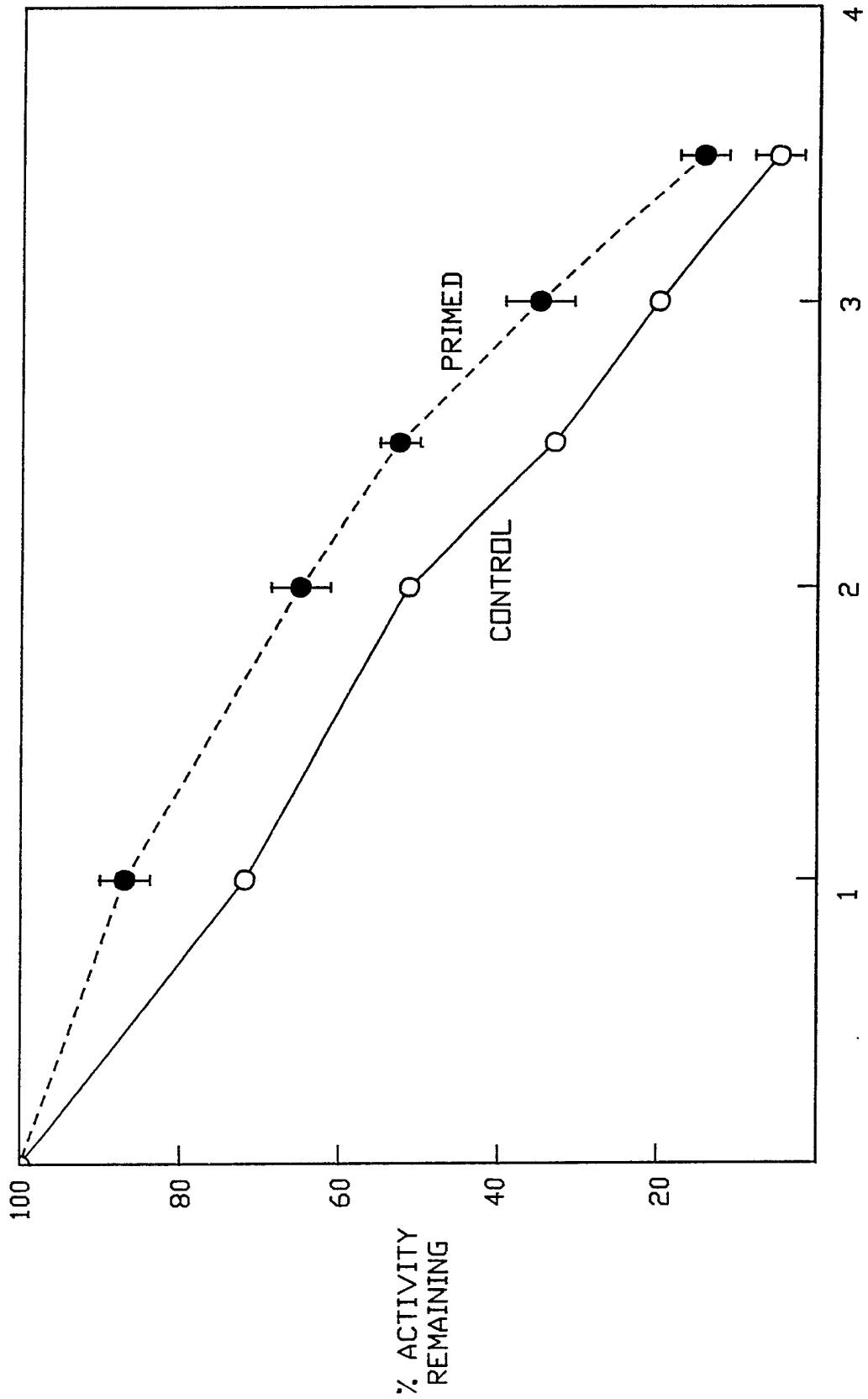

FIG.—5

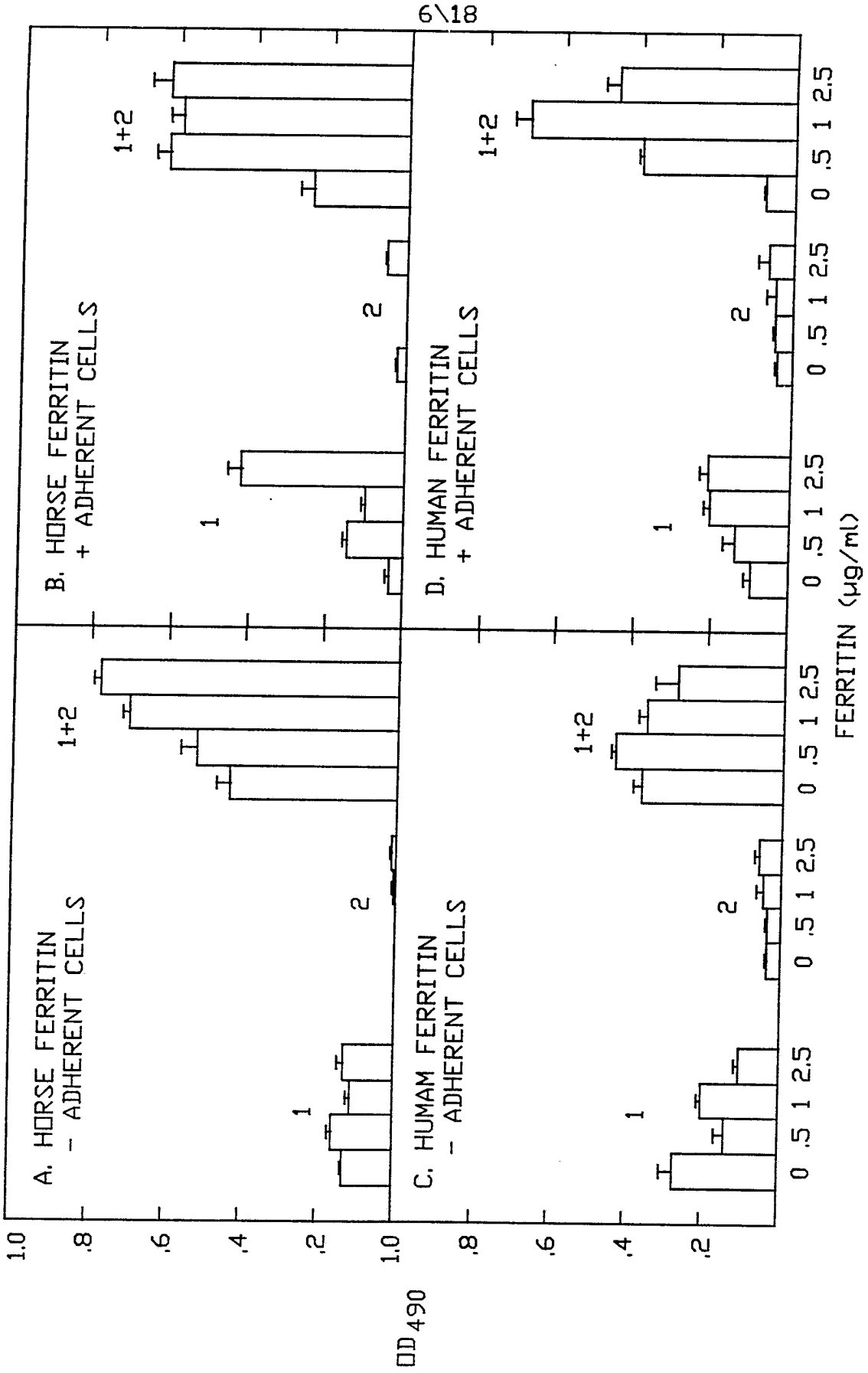
A. HORSE FERRITIN
- ADHERENT CELLS
B. HORSE FERRITIN
+ ADHERENT CELLS
C. HUMAM FERRITIN
- ADHERENT CELLS
D. HUMAN FERRITIN
+ ADHERENT CELLS
1
2
1+2
0 .5 1 2.5
FERRITIN (µg/ml)
OD 490
1.0
.8
.6
.4
.2

FIG.—6

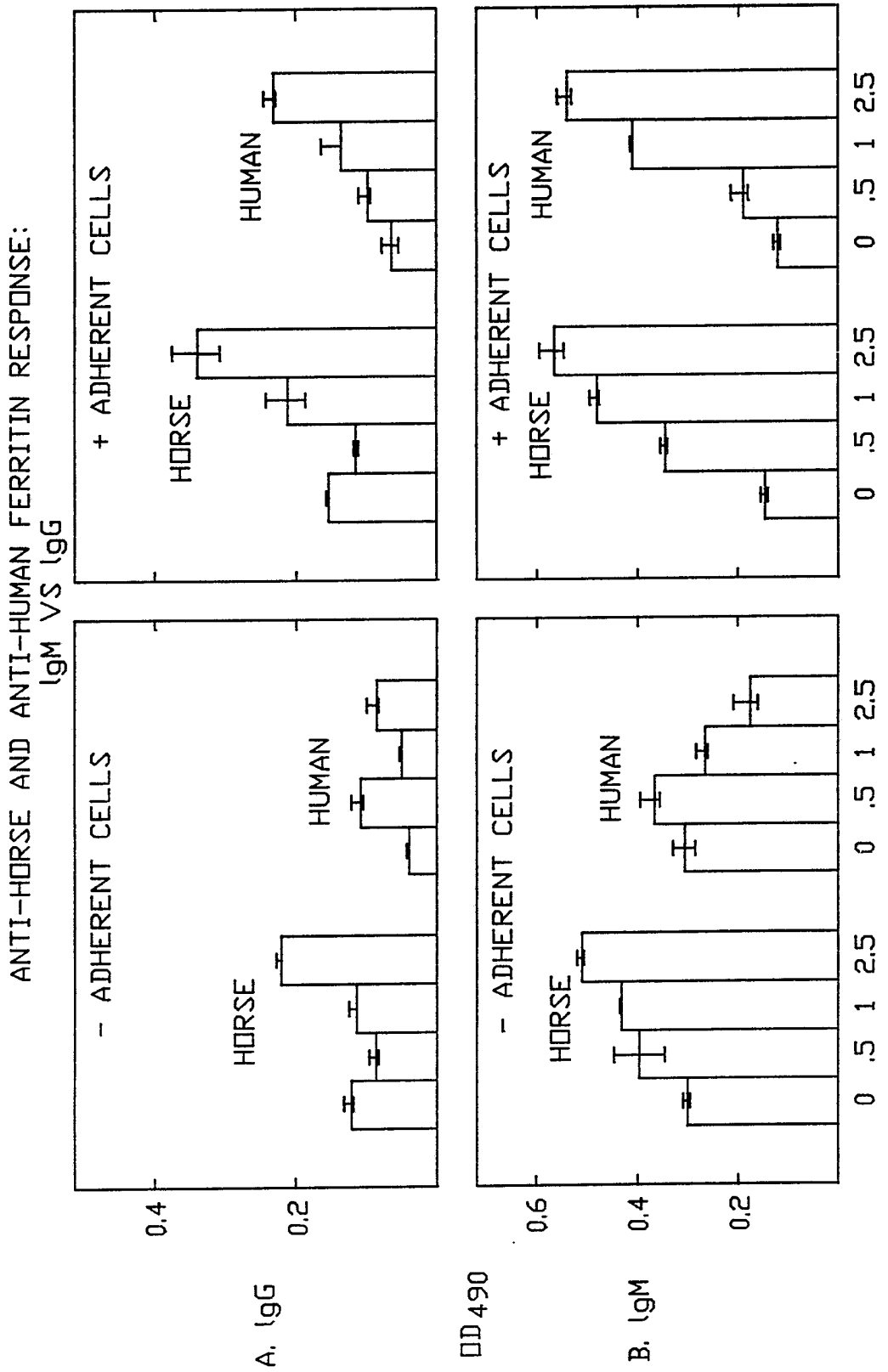
ANTI-HORSE AND ANTI-HUMAN FERRITIN RESPONSE:
IgM VS IgG
- ADHERENT CELLS
+ ADHERENT CELLS
HORSE
HUMAN
A. IgG
B. IgM
OD 490
0.4
0.2
0.6
0 .5 1 2.5
FERRITIN (µg/ml)

FIG. —7

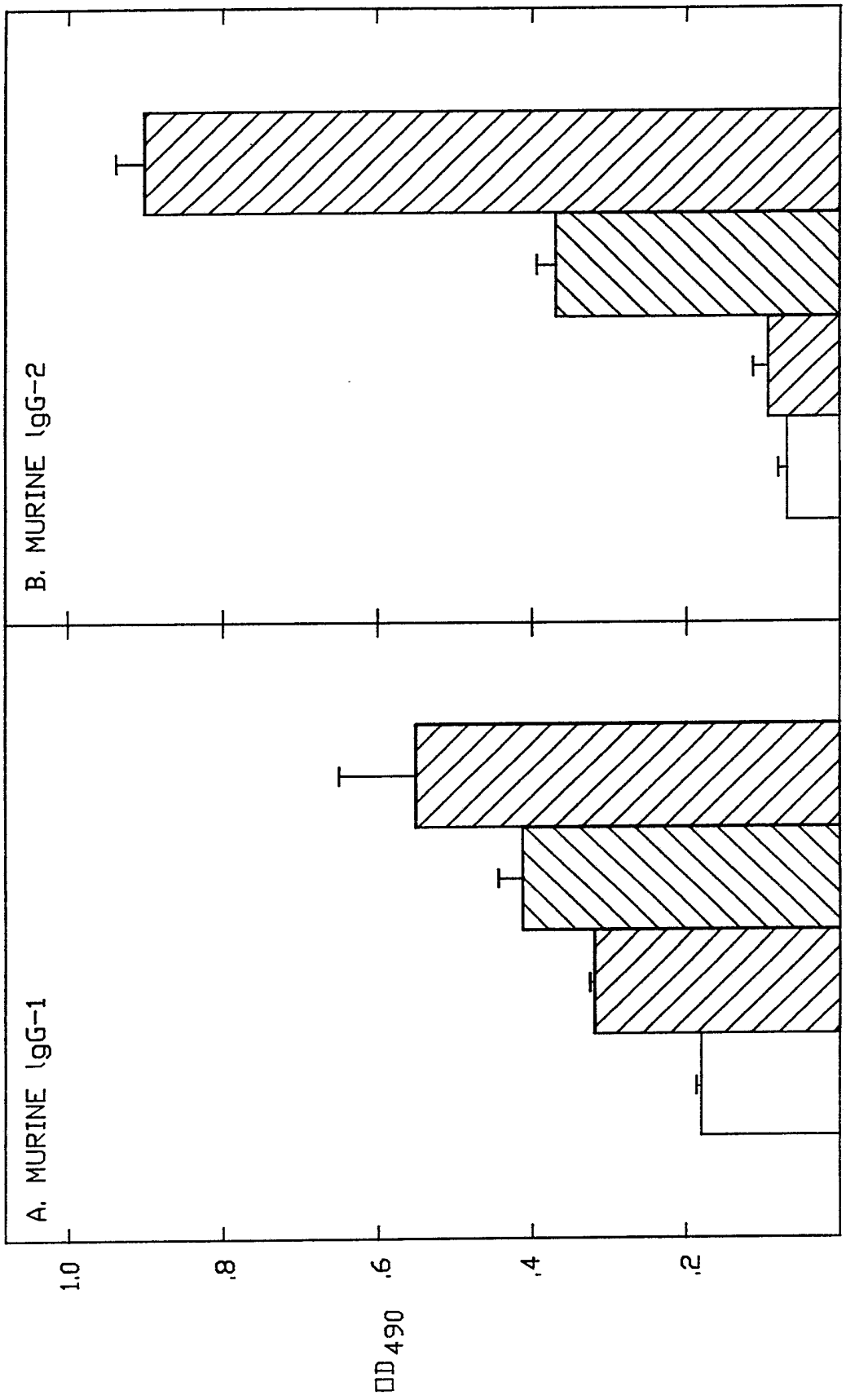
A. MURINE IgG-1
B. MURINE IgG-2
OD 490
1.0
.8
.6
.4
.2
0
.1
.5
2.0
MURINE MONOCLONAL ANTIBODY (µg/ml)

FIG.—8

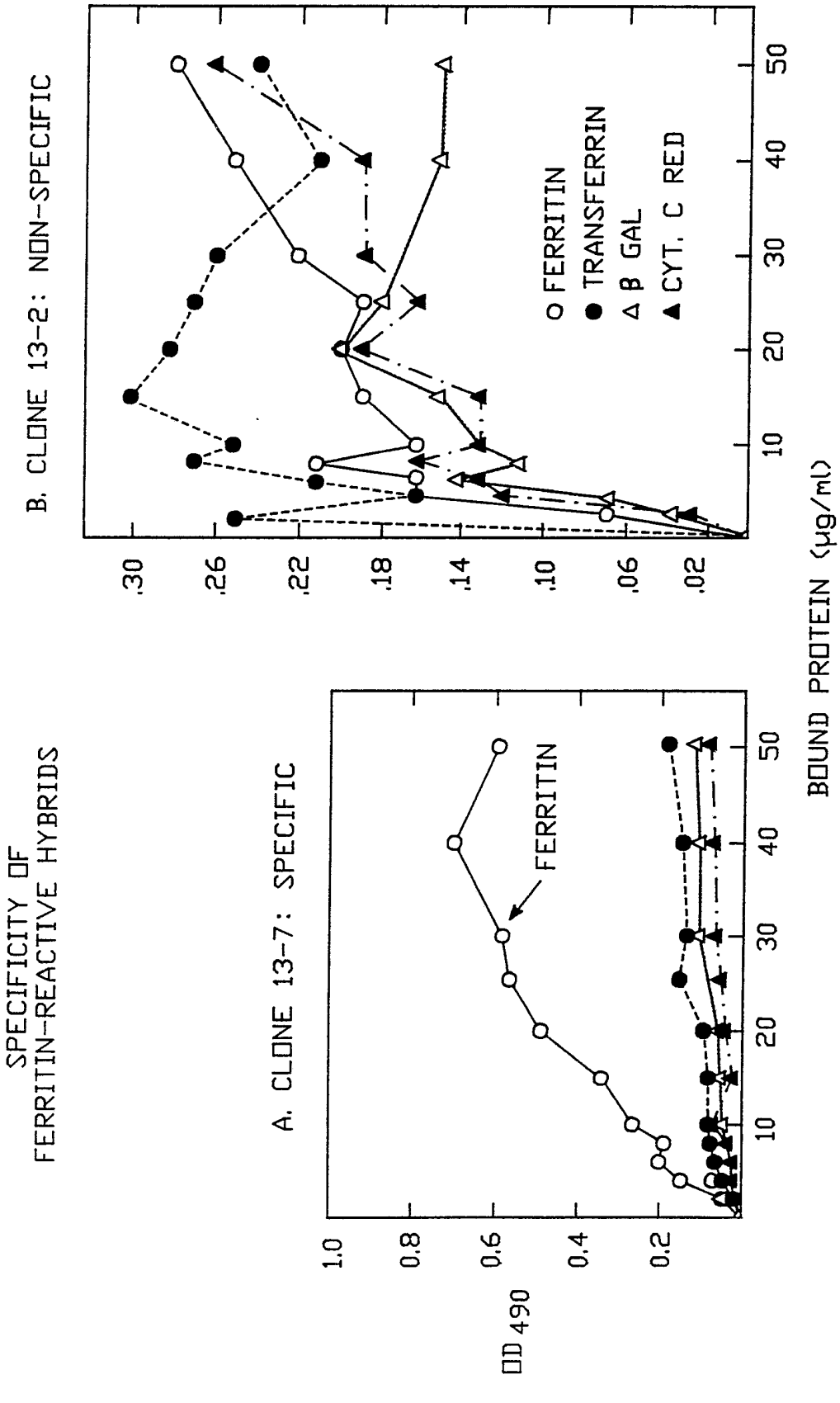
SPECIFICITY OF
FERRITIN-REACTIVE HYBRIDS
A. CLONE 13-7: SPECIFIC
FERRITIN
OD 490
1.0
0.8
0.6
0.4
0.2
10
20
30
40
50
B. CLONE 13-2: NON-SPECIFIC
O FERRITIN
● TRANSFERRIN
Δ β GAL
▲ CYT. C RED
.30
.26
.22
.18
.14
.10
.06
.02
BOUND PROTEIN (μg/ml)

FIG.—9

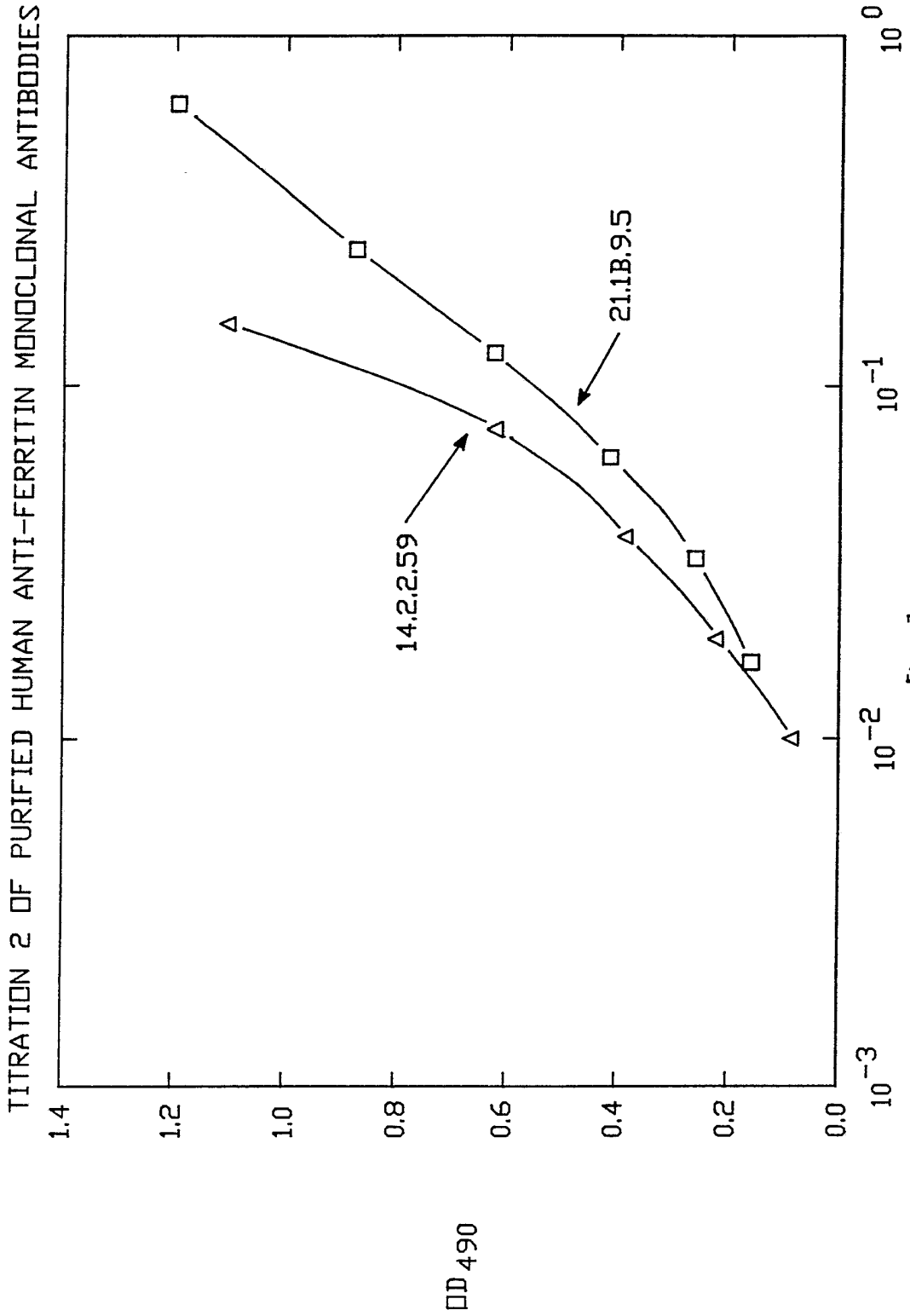
TITRATION 2 OF PURIFIED HUMAN ANTI-FERRITIN MONOCLONAL ANTIBODIES
21.1B.9.5
14.2.2.59
OD 490
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10^{-3}
10^{-2}
10^{-1}
10^{0}
[IgG] µg/ml

FIG.—10

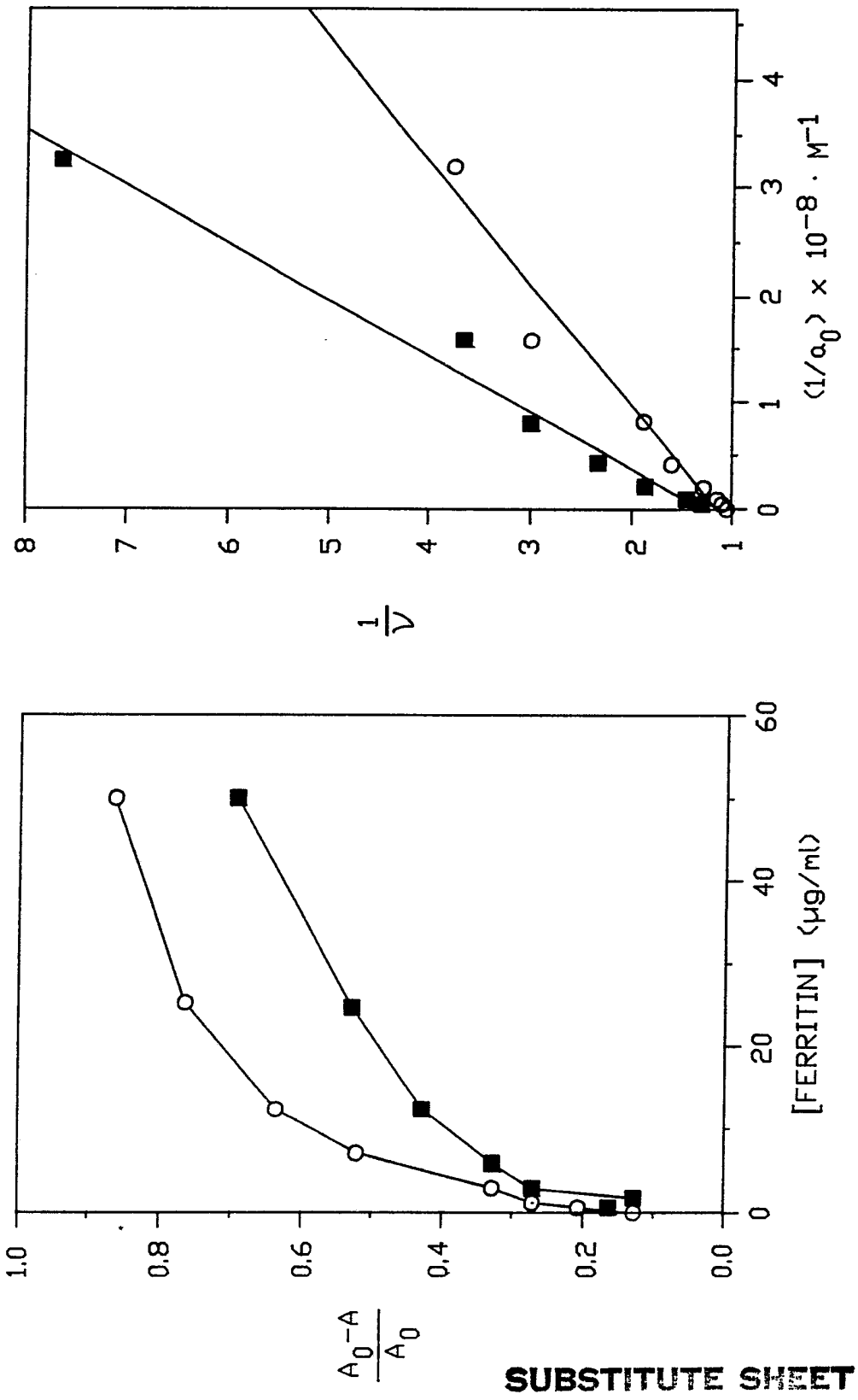
$\frac{1}{V}$
$(1/a_0) \times 10^{-8} \cdot M^{-1}$
0
1
2
3
4
1
2
3
4
5
6
7
8
$\frac{A_0 - A}{A_0}$
[FERRITIN] (µg/ml)
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60

FIG.—11

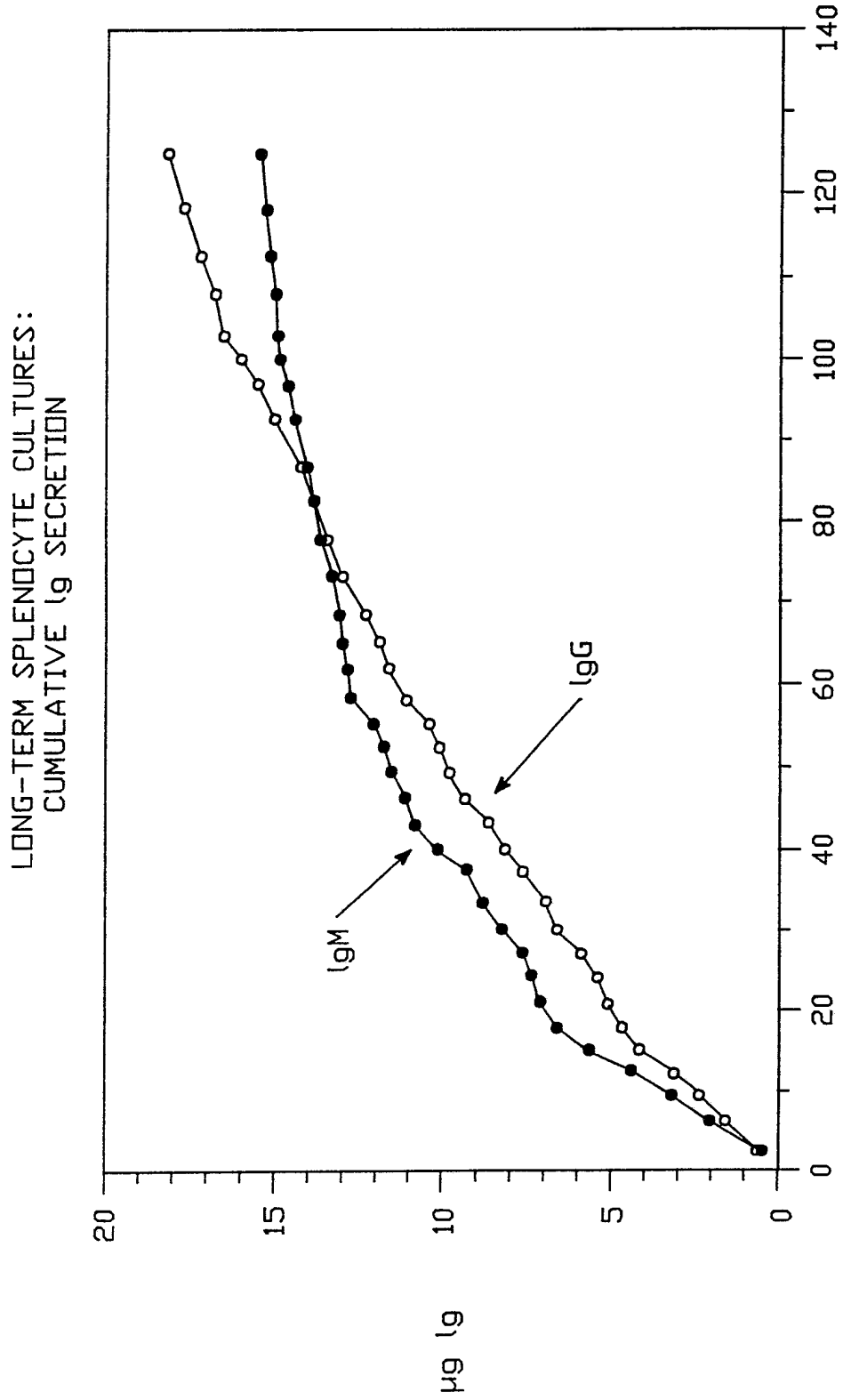
LONG-TERM SPLENOCYTE CULTURES:
CUMULATIVE Ig SECRETION
IgM
IgG
μg Ig
DAYS
0
5
10
15
20
0
20
40
60
80
100
120
140

FIG.—12

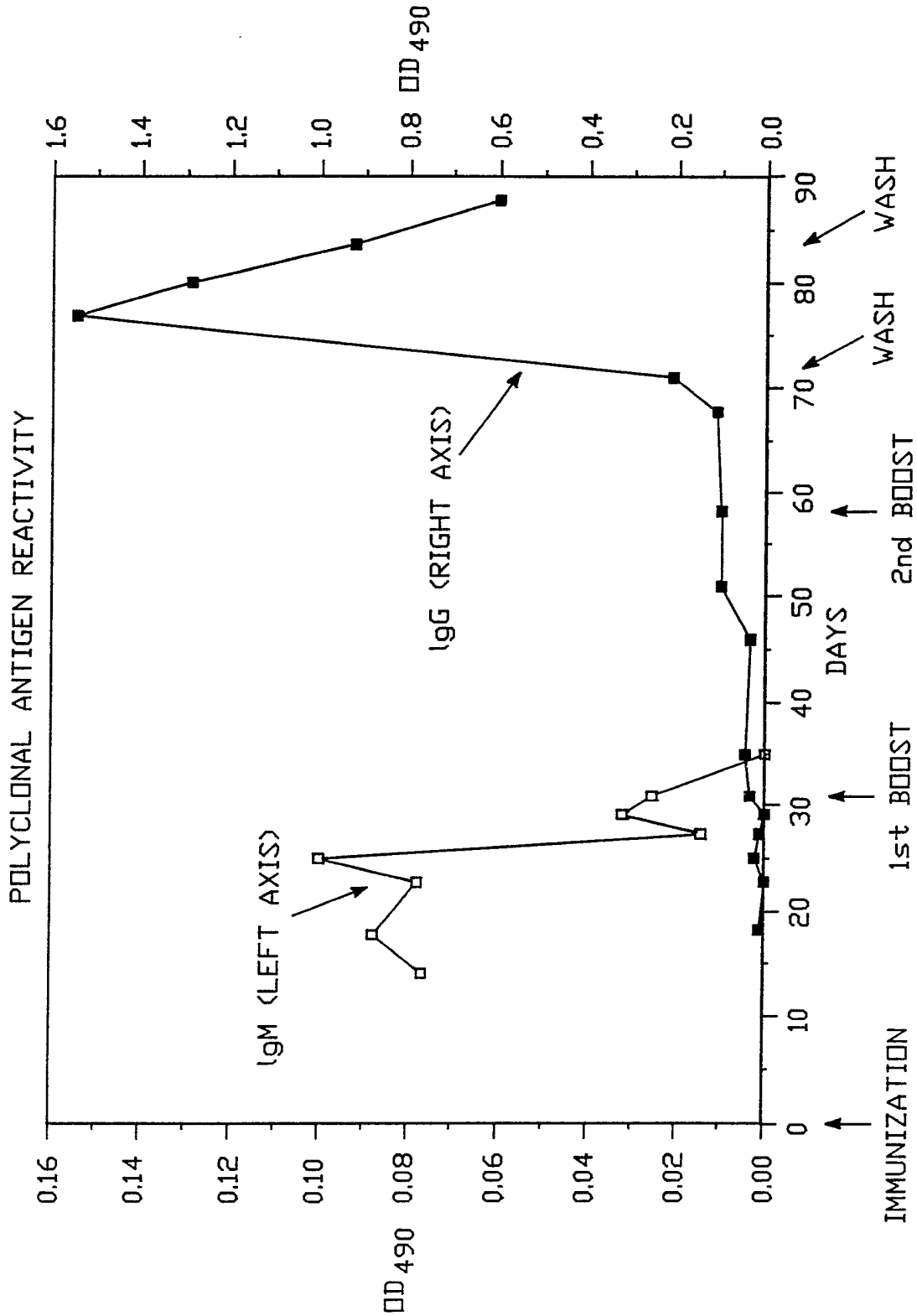
POLYCLONAL ANTIGEN REACTIVITY
IgM (LEFT AXIS)
IgG (RIGHT AXIS)
OD 490
OD 490
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
70
80
90
DAYS
IMMUNIZATION
1st BOOST
2nd BOOST
WASH
WASH
WASH

FIG.—13

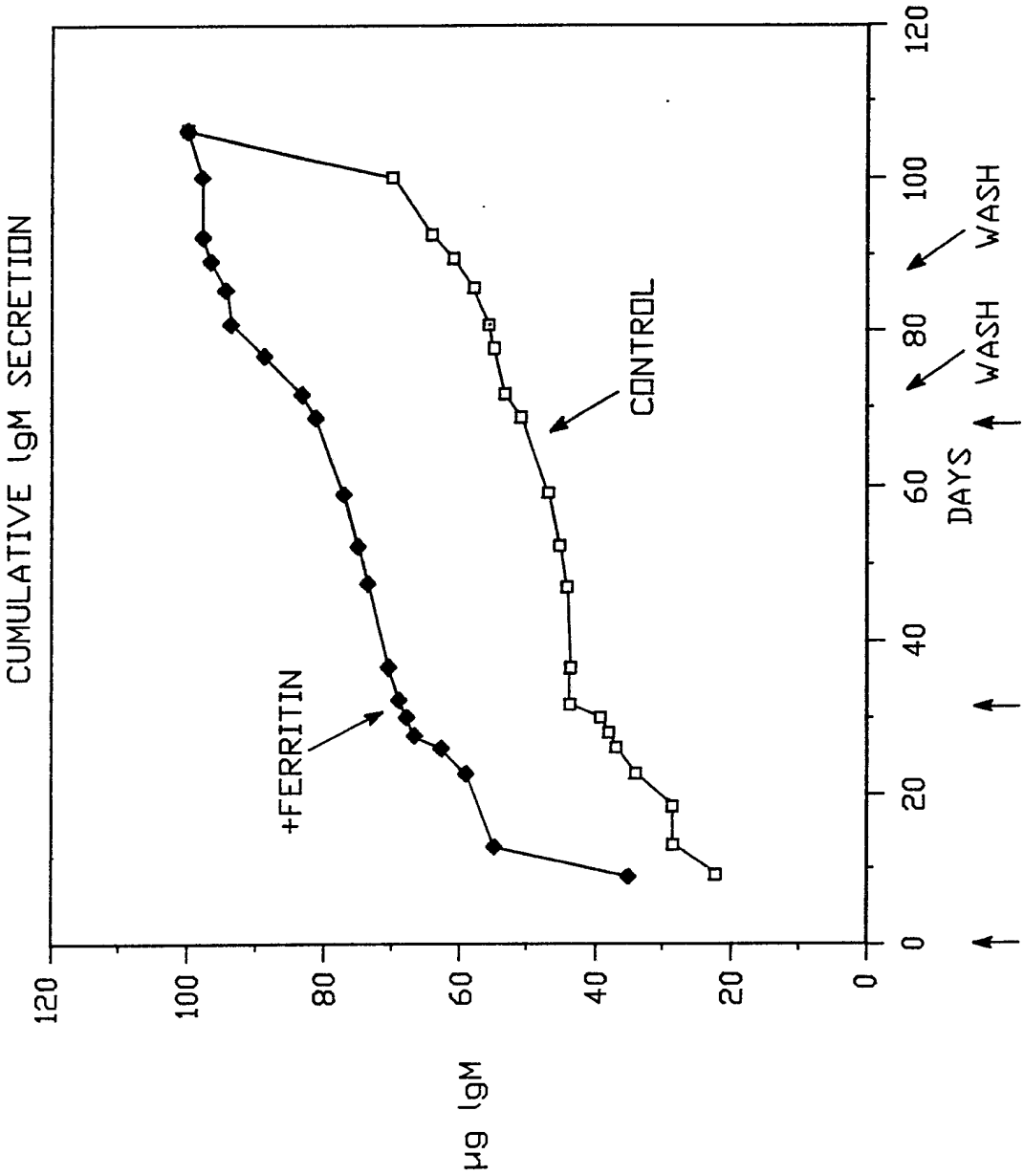
CUMULATIVE IgM SECRETION
μg IgM
120
100
80
60
40
20
0
+FERRITIN
CONTROL
IMMUNIZATION
1st BOOST
2nd BOOST
WASH
WASH
DAYS
0
20
40
60
80
100
120

FIG.—14

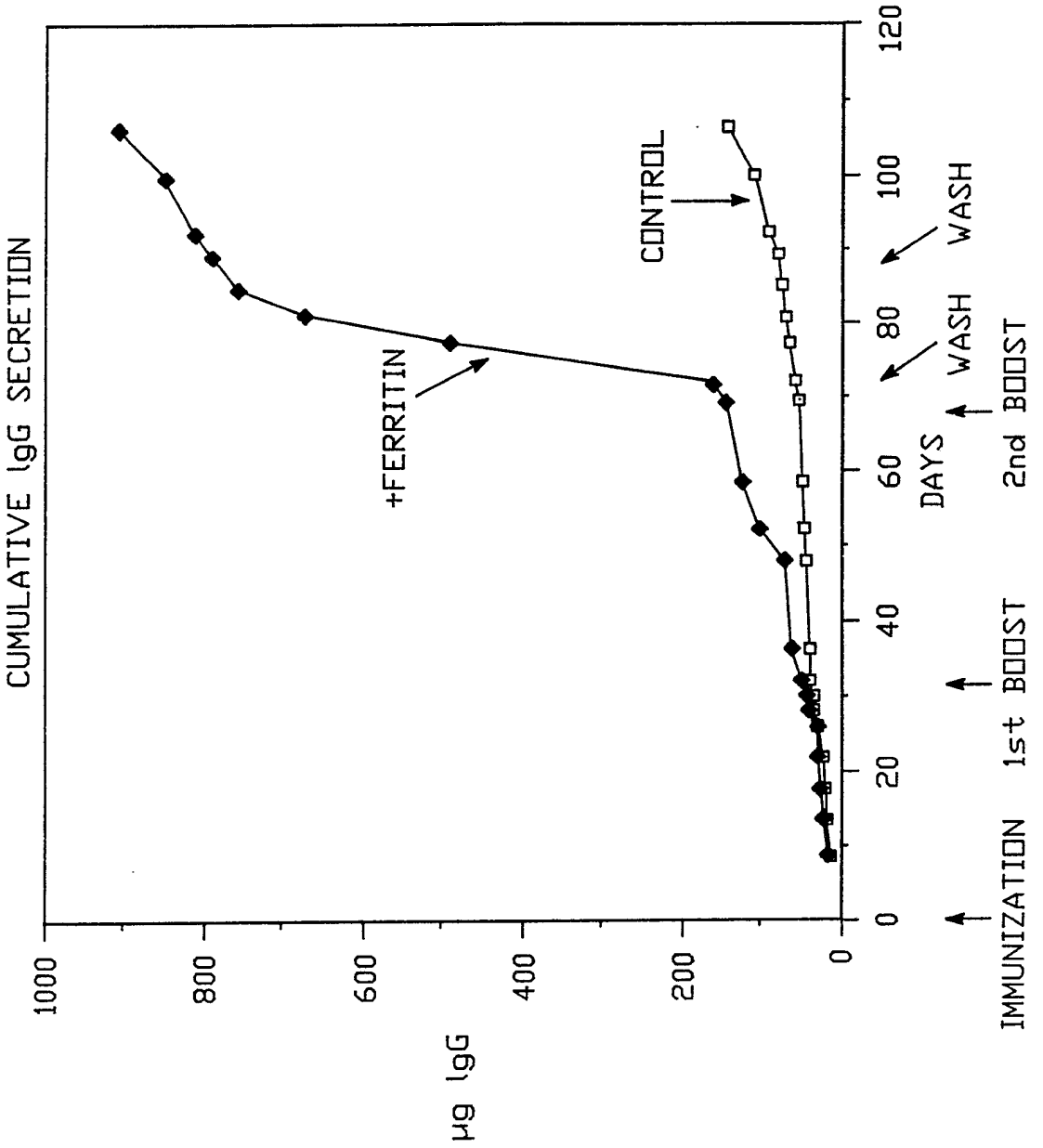
CUMULATIVE IgG SECRETION
μg IgG
1000
800
600
400
200
0
0
20
40
60
80
100
120
DAYS
IMMUNIZATION
1st BOOST
2nd BOOST
WASH
WASH
+FERRITIN
CONTROL

FIG.—15

FIG.—16

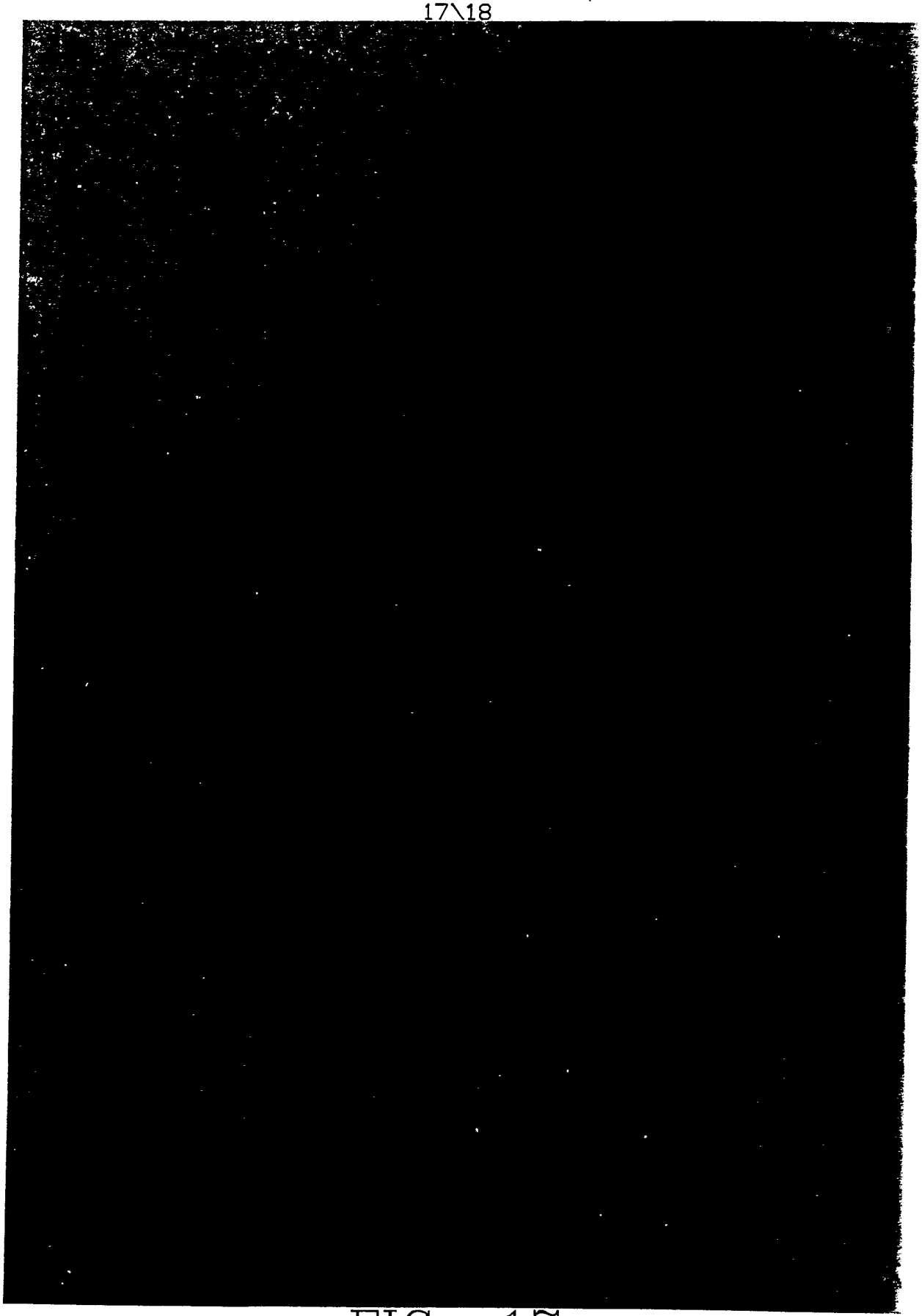

FIG.—17

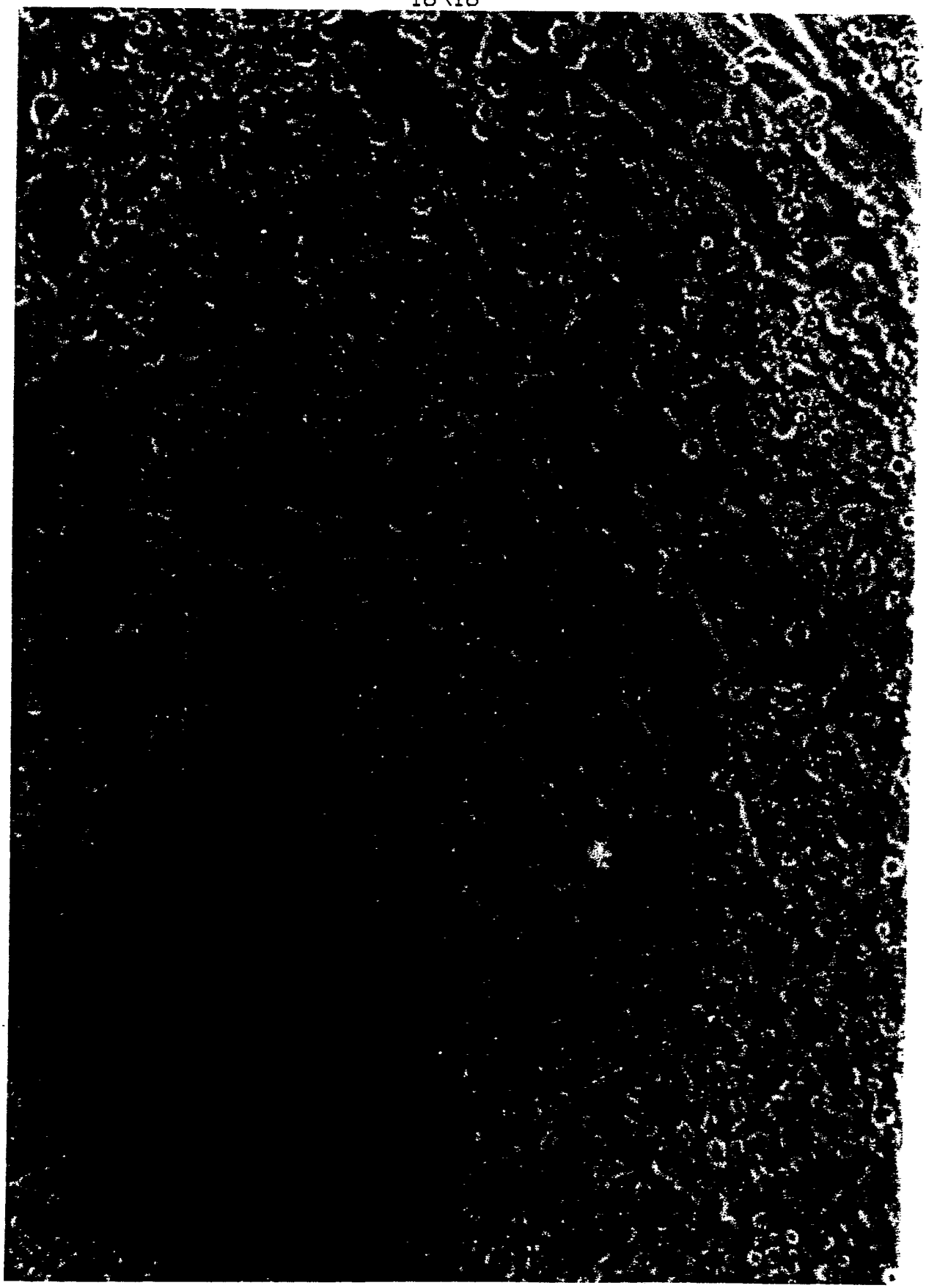

FIG.—18

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/03406

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/395

US Class: 424/85.8

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S	424/85.8; 435/240.2, 240.27, 70.21; 530/387

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Databases: Dialog (files: 5, 73, 155, 159, 76, 357, 399, 35, 77), USPTO Automated Patent Systems (file USPAT, 1971-1990).

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X,P	Proceedings of the 75th Annual Meeting of the Federation of American Societies for Experimental Biology, vol. 5, number 5, issued 15 March 1991, P. Brams et al., "In Vitro Immunization of human splenocytes: Repeated immunization enhances the antigenspecific IgG response," see abstract. No. 3584	1-9, 12-26
Y	US, A, 4,910,131 (Mellman et al) 20 March 1990. See the entire document, especially the abstract and column 9, lines 40-68 and column 10, lines 1-39.	33
X	Human Antibodies and Hybridomas, volume 1, number 1, Issued January 1990, Koda et al., "In Vitro Immunization for the Production of Human Monoclonal Antibody," pages 15-22, see the entire document.	1-30, 33
X, Y	UCLA Symposium of Molecular and Cellular Biology "Human Tumor Antigens and Specific Tumor Therapy", published 1989 by Alan R. Liss, Inc., see pages 147-156.	1-33

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
12 August 1991	19 SEP 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Susan L. Futrovsky

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
_X_ Y	US, A, 4,699,880 (Goldstein) 13 October 1987. See the entire document, especially column 4, lines 51-68 and column 5, lines 1-24.	33 1-9, 12-30.
Y	Immunochemica, volume II, Number 2, issued June 1988, Aquila, et al., "Class and Subclass Switching of Hybridomas in Vitro." page 1-4, see the entire document.	1-9 and 12-30
Y	European Journal of Biochemistry, volume 175, issued 1988, Boldicke et al., "Production of Specific Monoclonal Antibodies against the Active sites of Human Pancreatic Secretory Trypsin Inhibitor Variants by In Vitro Immunization with Synthetic Peptides", pages 259-264, see the entire document.	1-30
Y	Cancer Research, volume 48, issued 15 September 1988, Hsu, et al, "Establishment of Human Mesothelioma cell lines (MS-1,-2) and Production of a Monoclonal Antibody (anti-Ms with Diagnostic and Therapeutic Potential," pages 5228-5236, see the entire document especially page 5235.	1-32

DERWENT-ACC-NO: 1991-369008

DERWENT-WEEK: 199527

TITLE: In vitro human IgG prodn. by lymphocyte immunisation useful for diagnosis and therapy of cancer or viral or bacterial disease

INVENTOR: BOERNER P; ROYSTON I ; ROYSTON Y

PATENT-ASSIGNEE: UNIV CALIFORNIA[REGC]

PRIORITY-DATA: 1991US-688654 (April 19, 1991) , 1990US-527203 (May 22, 1990)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE
WO 9117769 A	November 28, 1991	EN
AU 9181070 A	December 10, 1991	EN
FI 9205081 A	November 9, 1992	FI
EP 533841 A1	March 31, 1993	EN
NO 9204475 A	November 20, 1992	NO
HU 62803 T	June 28, 1993	HU
JP 05507414 W	October 28, 1993	JA
AU 657482 B	March 16, 1995	EN
EP 533841 A4	July 28, 1993	EN

DESIGNATED-STATES: AU CA FI HU JP KR NO SU AT BE CH DE DK ES FR GB GR IT LU NL SE AT BE CH DE DK ES FR GB GR IT LI LU NL SE

APPLICATION-DATA:

PUB-NO	APPL-DESCRIPTOR	APPL-NO	APPL-DATE
WO1991017769A	N/A	1991WO-US03406	May 21, 1991
EP 533841A4	N/A	1991EP-912968	May 21, 1991
AU 657482B	N/A	1991AU-081070	May 21, 1991
EP 533841A1	N/A	1991EP-912968	May 21, 1991
JP 05507414W	N/A	1991JP-512269	May 21, 1991
FI 9205081A	N/A	1991WO-US03406	May 21, 1991
NO 9204475A	N/A	1991WO-US03406	May 21, 1991
EP 533841A1	N/A	1991WO-US03406	May 21, 1991
HU 62803T	N/A	1991WO-US03406	May 21, 1991
JP 05507414W	N/A	1991WO-US03406	May 21, 1991
HU 62803T	N/A	1992HU-003635	May 21, 1991
FI 9205081A	N/A	1992FI-005081	November 9, 1992
NO 9204475A	Based on	1992NO-004475	November 20, 1992

INT-CL-CURRENT:

TYPE	IPC DATE
CIPP	A61K39/395 20060101
CIPS	C07K16/00 20060101
CIPS	C07K16/18 20060101
CIPS	C07K16/42 20060101
CIPS	C12N15/02 20060101
CIPS	C12N5/00 20060101
CIPS	C12N5/02 20060101
CIPS	C12N5/06 20060101
CIPS	C12N5/08 20060101
CIPS	C12N5/10 20060101
CIPS	C12N5/16 20060101
CIPS	C12N5/24 20060101
CIPS	C12P21/08 20060101
CIPS	C12P21/08 20060101
CIPN	C12R1/91 20060101

ABSTRACTED-PUB-NO: WO 9117769 A

BASIC-ABSTRACT:

An in vitro immunisation culture (I) of human lymphocyte derived lymphoid tissue, pref. spleen or splenocytes has essential numbers of autologous accessory cells immunised with a specific antigen in the absence of growth factors, and produces IgG (human) monoclonal antibodies (II) with antigen binding affinity of 50 million 1/mol. (II) are claimed together with: (1) their method of production via in vitro immunisation of lymphocytes (specifically splenocytes) with multiple macroscopic aggregate forms (2) their labelled, esp. yttrium-90 or indium-111 radiolabelled forms, (3) their producing hybridomas and stable continuous cell cultures, and (4) a method

for diagnosis and therapy of cancer and viral or bacterial diseases using the labelled monoclonal antibodies. (I) is esp. an allogenic coculture contg. multiple splenoids, and is pref. immortalised by cell fusion to form a hybridoma or by amplification of variable regions of the antibody.

USE/ADVANTAGE - The in vitro immunisation method produces sufficient quantities of efficacious human monoclonal antibodies for diagnosis and therapy of disease e.g. tumours.

TITLE-TERMS: VITRO HUMAN IMMUNOGLOBULIN PRODUCE LYMPHOCYTE IMMUNE USEFUL DIAGNOSE THERAPEUTIC CANCER VIRUS BACTERIA DISEASE

DERWENT-CLASS: B04 D16 K08

CPI-CODES: B04-B04A3; B04-B04C2; B04-B04C5; B04-B04D1; B11-C08E1; B12-A01; B12-A06; B12-G07; B12-K04A; D05-H08; D05-H11; K09-B; K09-E;

CHEMICAL-CODES: Chemical Indexing M1 *01* Fragmentation Code A349 A539 A960 C811 C812 M423 M710 N136 P210 P220 P633 P831 Q233 Q444 V600 V611 V754 V791

SECONDARY-ACC-NO:

CPI Secondary Accession Numbers: 1991-159025